Characterization of diverse germplasm of sainfoin (*Onobrychis viciifolia* Scop.) using agro-morphological traits and AFLP molecular markers

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Abstract

Sainfoin (*Onobrychis viciifolia* Scop.) is a perennial forage legume, widely distributed in northern temperate regions of the world. Unlike alfalfa (*Medicago sativa* L.), sainfoin does not cause bloat in grazing animals due to the presence of condensed tannins. However, this species is still an underdeveloped legume, with limited information available on genetic diversity, agronomic and phenotypic characteristics. The objectives of this study were: 1) to evaluate genetic variation and relationships among 38 sainfoin accessions using amplified fragment length polymorphism (AFLP) markers; 2) to measure agro-morphological traits and nutritive value of the 38 sainfoin accessions; 3) to understand the effects of seed size, seed pod removal and temperature on sainfoin seed germination. A field plots of 38 sainfoin accessions were planted in July 2014 using a randomized complete block design with four replications near Saskatoon, SK, Canada. Five AFLP primer pairs were employed to genotype 367 plants and 1,042 polymorphic AFLP bands were detected. The analysis of molecular variance (AMOVA) revealed that 84.1% of the total molecular variation was present within accessions, while 15.0% and 0.9% of the variation resided among accessions and regions (continents), respectively. Data were collected in the growing seasons of 2015 and 2016. An analysis of variance (ANOVA) revealed significant variations for all measured traits among the accessions ($P<0.05$). The 2-yr mean data revealed winter survival of sainfoin accessions ranged from 20–94%. Similarly, accessions showed a wide range of plant height from 37–70 cm. Forage dry matter (DM) yield ranged from 74–239 g plant^{-1} and seed yield ranged from 5–64 g plant^{-1}. Similarly, wide variations were observed for nutritive values. Forage DM yield had a positive correlation with plant height (r=0.82, $P<0.001$), stem number (r=0.75, $P<0.001$), and 1000-seed weight (r=0.29, $P<0.001$), whereas negative correlations were found with days to flower (r=−0.57, $P<0.001$) and crude protein (CP) concentration ($r=-0.62$, $P<0.001$) were observed. Based on agronomic performance and nutritive value traits, the accessions were grouped into three major clusters. Seed germination of sainfoin varied significantly ($P=0.047$) at different temperatures with the highest final germination at 20/10°C (day/night temperature) and 15/5°C temperatures. Seed pod removal significantly ($P<0.001$) enhanced seed germination. The seed germination experiments suggested the possibility of decreasing seed size without affecting germination percentage of sainfoin seed within the same accession. In conclusion, the information obtained from this study on agro-morphological traits, nutritive values and genetic diversity among the 38 sainfoin accessions will be useful for future sainfoin breeding programs.

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Dedication

To my parents

&

To all of my teachers including my lifelong teacher my brother

for making me what I am today.

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List of Abbreviations

Abbreviation	
ADF	Acid Detergent Fiber
AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
CP	Crude Protein
DM	Dry Matter
DNA	Deoxyribonuclei acid
ISSR	Inter-Simple Sequence Repeats
LSD	Least Significant Differences
NDF	Neutral Detergent Fiber
NJ	Neighbor Joining
PCA	Principal Component Analysis
PCoA	Principal Coordinates Analysis
PIC	Polymorphism Information Content
PLS	Pure Live Seed
RAPD	Random Amplified Polymorphic DNA
SNP	Single Nucleotide Polymorphism
UPGMA	Unweighted Pair-group Method with Arithmetic mean

Chapter 1. Introduction

There are approximately 11.9 million cattle in Canada, generating a significant return to the Canadian economy (Canfax Research Services 2016). In Saskatchewan, the beef industry is one of the major sectors of agriculture, with close to 2.3 million cattle (Canfax Research Services 2016). Approximately 80% of Canada's beef production depends on forages as the main feed source (Canfax Research Services 2014). There is an increasing demand for high quality forage crops, particularly non-bloating legumes for grazing. The occurrence of pasture bloat is a major constraint for use of the important forage legumes, such as alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.), for grazing. Pasture bloat is a digestive disorder in cattle marked by a collection of gas in the rumen that the animal is unable to expel (Lees 1992). Annual cattle loss from grazing alfalfa pasture in Canada is valued at $30–50 million (Acharya et al. 2013).

Sainfoin (*Onobrychis viciifolia* Scop.) is a perennial legume with erect or sub-erect, hollow stems. The inflorescence of sainfoin has up to 70–80 pink flowers in an erect raceme producing single-seeded indehiscent pods (Figure 1.1 A–E). It is a valuable forage for beef producers because it is a non-bloating legume with high condensed tannin content and high forage dry matter yield (Goplen et al. 1991; Wang et al. 2015). Sainfoin is adapted to the growing conditions of Western Canada and it is a highly palatable and nutritious forage legume for both hay and pasture production (Goplen et al. 1991). In recent years, grazing sainfoin either as a monoculture stand, or in mixtures with perennial grasses or alfalfa, is becoming a popular practice in North America and Europe (Frame et al. 1998; Hayot-Carbonero et al. 2011). Condensed tannins present in sainfoin bind to protein in alfalfa, which can prevent pasture bloat in alfalfa-sainfoin pasture (Jones et al. 1976; McMahon et al. 2000; Waghorn and McNabb 2003; Waghorn 2008).

Although sainfoin was introduced to North America in 1786 (Hanna 1972), it is a less developed legume with only three cultivars released in Canada. The number of germplasm available for sainfoin breeding is limited in Canada. New germplasm introduction from gene banks is important for expanding genetic resources available to future breeding of this crop. Therefore, a comprehensive study to examine phenotypic and agronomic characteristics and genetic variation of introduced and existing germplasm is important to select desirable breeding accessions for sainfoin breeding programs. It is hypothesized that: 1) sainfoin accessions are genetically diverse, but the accessions from similar geographical region are genetically related; 2) agro-morphological characteristics and nutritive values differ among sainfoin accessions, but accessions adapted to the North America have superior agronomic performance; 3) seed pod removal and larger seed size will increase germination rate under different temperatures. The objectives of the study are: 1) to evaluate genetic variation and relationships among 38 sainfoin accessions using amplified fragment length polymorphism (AFLP) markers; 2) to measure agro-morphological traits and nutritive value of the 38 sainfoin accessions; 3) to understand the effects of seed size, seed pod removal and temperature on sainfoin seed germination.

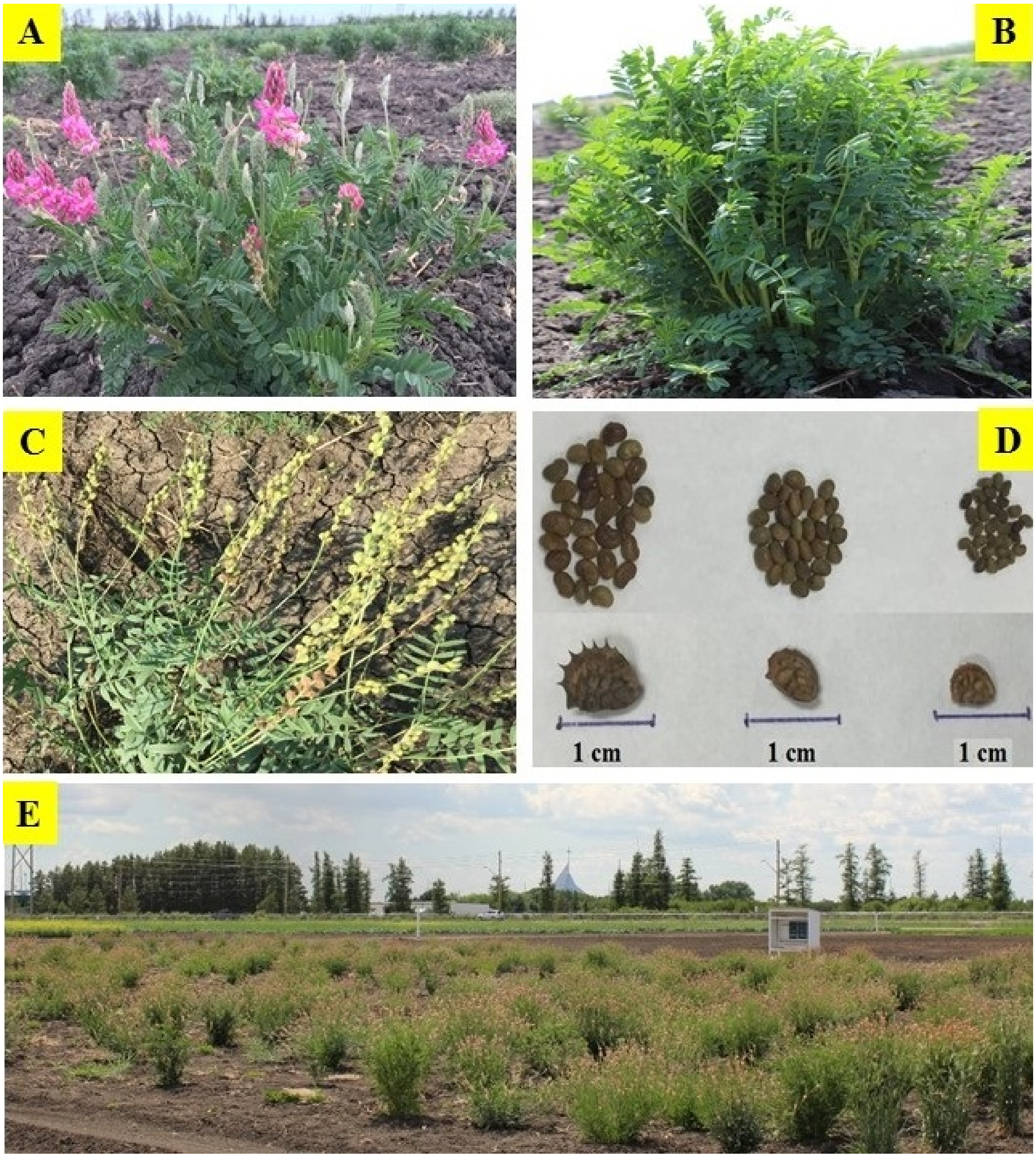

Figure 1.1 A) sainfoin flower, B) sainfoin plant, C) seed setting in sainfoin, D) sainfoin seed, and E) sainfoin research plot at Saskatoon.

Chapter 2. Literature review

This chapter with some modifications has been published in the Canadian Journal of Plant Science.

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2.1 Origin and Description

Sainfoin (*Onobrychis viciifolia* Scop.) is native to south central Asia (Burton and Curley 1968). It has been cultivated for many years in Russia, Europe and many parts of Asia (Melton 1973). Sainfoin was introduced into North America in 1786 (Hanna 1972). Sainfoin has two agricultural types: common type (*O. sativa* var. *communis* Ahlefed) and giant type (*O. sativa* var *bifera* Hort.). Thomson (1938) found that the common type is long-lived, but less vigorous in the establishment year, whereas the giant type is short lived in the United Kingdom. Compared to the giant type, the common type has more stems per plant, but plant height is generally shorter (Hayot-Carbonero et al. 2011). In Europe, the common type is also known as a single-cut type, while the giant type is used for a double-cut system. The common type originates from central Europe and the giant type is from the Middle East. Introductions from Western Europe, mainly the giant type, were low-yielding and poorly adapted to North America; however, introductions of the common type from the former Soviet Union and Turkey performed relatively well under Western Canadian growing conditions (Goplen et al. 1991). The first Canadian sainfoin cultivar, cv. Melrose, released in 1969, was selected from accessions introduced from the former Soviet Union (Hanna et al. 1970).

Sainfoin is a deep-rooted perennial legume with erect or sub-erect hollow stems growing up to a height of 100 cm (Frame et al. 1998). It has a deep tap root system with few main branches and numerous fine lateral roots, which provide sites for root nodule development. Leaves are pinnately compounded with 5 to 14 pairs of leaflets and a terminal leaflet.

Sainfoin shows an indeterminate growth habit with the flowering period starting in early June and lasting about 60 days in Western Canada (Goplen et al. 1991). Sainfoin axillary tillers possess inflorescences in an erect raceme, which contains approximately 70–80 flowers. Pink, white or purple flowers of sainfoin are highly attractive to pollinators. Sainfoin is generally cross-pollinated, mostly by honey bees (*Apis mellifera* L.) and leaf cutting bees (*Megachile rotundata* Fabricius.) (Goplen et al. 1991). In addition, bumble bees (*Bombus* spp.) are also frequent visitors to sainfoin fields. Pollination success is relatively high in sainfoin. The duration between the opening of the first flower and the withering of the terminal flowers on a raceme is around 2–3 weeks. Sainfoin produces single-seeded indehiscent brown colored pods. Seed color varies from olive to brown or black (Goplen et al. 1991).

2.2 Adaptation

Sainfoin is adapted to a wide range of climatic and soil conditions and is cultivated from hot and dry Mediterranean regions to northern latitudes with severe winters. It can grow well in neutral to slightly alkaline soil (Frame 2005), but it does not tolerate high soil salinity and high water tables (Sheldrick et al. 1987). It is fairly drought tolerant because of its deep tap root; however, sainfoin dry matter (DM) yield and stand density may reduce dramatically after several years of drought (Biligetu et al. 2014). Ditterine and Cooper (1975) recommended that sainfoin be seeded in areas with rainfall amounts of 330 mm or higher. Meyer and Badaruddin (2001) found that sainfoin seedlings have relatively high frost tolerance. The sainfoin cultivars developed in Canada have high winter survival (Goplen et al. 1991) however, proper stand management is still considered a key factor to reduce winter kill in Western Canada. Brown, Dark Brown and Black soils are best for sainfoin, however, performs poor in Gray Wooded and Light Brown soils of Western Canada (Goplen et al. 1991).

2.3 Seed and Seed Production

Goplen et al. (1991) suggested 20,000 leaf cutting bees in a hectare of sainfoin are sufficient for successful seed production. The challenge with sainfoin seed production is the lack of uniform maturity due to its indeterminate growth form. Research conducted at six different locations in Western Canada showed that the seed yield of the cultivar Melrose ranged from 307–1531kg ha^{-1} (Hanna et al. 1972).

Sainfoin is generally available as either “unmilled seed”, which has adhering seed pods, or “milled seed” without pods. The size of sainfoin seed varied among germplasm accessions with seed length ranging from 2.5–4.5 mm, and seed width from 2–3.5 mm (Thomson 1951). The 1000-seed weight is about 15 g (milled) and 24 g (unmilled) (Hayot-Carbonero et al. 2011), which is approximately six times larger than alfalfa seed. Also, Thomson (1951) reported 1000-seed weight of 15.3 g (milled) and 21.4 g (unmilled) for the giant type, and 15.7 g (milled) and 21.2 g (unmilled) for the common type. Thus, the seed size is similar between the two types of sainfoin. The large seed size of sainfoin results in high seed costs for stand establishment.

Carleton et al. (1968) reported an optimum germination temperature of 15 to 20°C for sainfoin seed. There are conflicting reports on the relative germination of milled and unmilled sainfoin seeds. Wiesner et al. (1968) reported a higher germination percentage for milled seeds, but no significant difference in germination among the two types was observed by Chen (1992). Noorbakhshian et al. (2011a) found improved germination and seedling vigor for sainfoin after removing the seed pods. The germination percentage was higher for brown seed than green seed, as the former is physiologically mature (Thomson 1951; Noorbakhshian et al. 2011a). Germination is also affected by seed size, which varies among sainfoin cultivars. The small seed class showed a high degree of physical dormancy. New sainfoin cultivars with smaller seed size will reduce the cost of stand establishment; however, a small seed class (1000-seed weight of 12 g or smaller) may reduce germination percentage. Cash and Ditterline (1996) reported that sainfoin seedlings emerged more rapidly from large seeds.

2.4 Diseases and Pests

Sainfoin is relatively resistant to a number of diseases and insects that can potentially damage alfalfa (Goplen et al. 1991). However, fungal diseases are the most common diseases in sainfoin (Mathre 1968). Crown and root rots caused by fungi, such as *Sclerotinia* or *Fusarium* species, may reduce the stand longevity of sainfoin in temperate climates (Mathre 1968). *Fusarium* species are soil borne pathogen causing *Fusarium* rot, characterized by the presence of dark brown to black, dry, necrotic lesions spreading into vascular tissue from the center of affected crowns and roots (Eken et al. 2004). Other fungal diseases of sainfoin include leaf spot, ring spot, leaf and stem spot, rust, chocolate spot and powdery mildew (Mathre 1968).

In addition to fungal diseases, a number of insect and nematode species damage sainfoin stands and reduce seed production in Canada. Weevils from the genus *Sitona* are root feeding insects and may reduce the persistence of sainfoin plants (Morrill et al. 1998). In some localized areas in Canada, potato leaf hopper (*Empoasca fabae* Harris.) is one of the most damaging pests of sainfoin, reducing protein concentration of the forage. Potato leaf hopper generally causes yellow triangles on the leaves. Various species of *Lygus* bug also feed on buds, flowers and seeds of sainfoin, but the insects do not cause major damage. The root-knot nematode (*Meloidogyne* spp.) has been found in sainfoin fields in the USA (Mathre 1968). The cultivar Shoshone, which is resistant to northern root-knot nematode (*Meloidogyne hapla* Chitwood.), was released in 2006 in Wyoming, USA (Gray et al. 2006). Sainfoin is resistant to alfalfa weevil, and it can be an alternative forage legume to alfalfa in areas where alfalfa weevil causes severe damage. In seven years of field study in Montana, USA, Morrill et al. (1998) found no or few alfalfa weevils and pea aphids (*Acyrthosiphon pisum* Harris.) in sainfoin fields. Alfalfa seed chalcid (*Bruchophagous* spp.), a seed infesting insect, has also been reported on sainfoin (Morrill et al. 1998). Seed weevil (*Apion* spp.) infestation has been observed in British Columbia, Canada (Hanna et al. 1972). The seed weevil is not a major pest, but its presence in the province of Saskatchewan has been recorded.

2.5 Agronomic Management

Ease of stand establishment is considered an important trait in perennial forage utilization. Sainfoin seeds generally germinate rapidly and are easy to establish in Brown, Dark Brown and Black soil zones of Western Canada (Goplen et al. 1991).

The seeding rate of sainfoin depends upon seed size, soil type, soil moisture, purpose and method of seeding. There is almost no research data available to confirm the effect of sainfoin seeding rate on stand establishment, forage yield, and other agronomic performance. Thus, the recommended seeding rate of sainfoin varied largely among sites. In Alberta, Canada, the recommended seeding rate for areas with annual precipitation not exceeding 400 mm is 14 kg ha^{-1} of pure live seed (PLS) (Government of Alberta Bulletin 2014). However, the recommended seeding rate of sainfoin for the Black and Grey Wooded soil zones in Saskatchewan is 33 kg ha^{-1} PLS (Saskatchewan Forage Crop Production Guide 2007). Goplen et al. (1991) recommended a seeding rate of 7 kg ha^{-1} for seed production, and 40 kg ha^{-1} for hay production, respectively in Saskatchewan, Canada. In the United States, sainfoin is usually planted in spring at a seeding rate of 38 kg ha^{-1} for pasture production, and 2.5–5.5 kg ha^{-1} in a rangeland seed mixture (USDA 2015). In Europe, Koivisto and Lane (2001) recommended 50 kg ha^{-1} of milled seed or 120 kg ha^{-1} of unmilled seed for sainfoin monoculture in the United Kingdom. Recommended seeding depth in Canada varied slightly between soil zones, but it is recommended to seed sainfoin no deeper than 2 cm (Goplen et al. 1991). Sainfoin requires the F type (*Rhizobium leguminosarum bv.viciae*) of seed inoculant, which differs from the alfalfa inoculant type. Sainfoin can be cross-inoculated by *Rhizobium* species recommended for sweetvetch (*Hedysarum* sp.), purple prairie clover (*Dalea purpurea* Vent.) and white prairie clover (*Dalea candida* Michx.) (Burton and Curley 1968). Koivisto and Lane (2001) recommended USDA 3291, a strain of *Rhizobium* spp., as an effective inoculant for sainfoin in the United Kingdom.

Sainfoin is considered to be a non-aggressive crop during seedling establishment. Thus, weed control in the first year is important for good establishment and high forage production in subsequent years. In the first year of establishment, Moyer (1985) found weeds made up 98% of DM yield in sainfoin fields without any weed control measures. In the year of establishment, Goplen et al. (1991) reported that the most abundant weeds in sainfoin fields were either winter annual species or kochia (*Kochia scoparia* L.) in Western Canada. In poorly managed older sainfoin stands, perennial weeds such as dandelion (*Taraxacum officinale* F.H. Wigg), sowthistle (*Sonchus arvensis* L.) and Canada thistle (*Cirsium arvense* (L.) Scop.) often reduce sainfoin DM yield.

Dandelion, a major weed in sainfoin pastures, is effectively controlled by the herbicides Hexazione [active ingredient 3-cyclohexyl-6-dimethylamino-1-methyl-1,3,5-triazine-2,4(1H,3H)-dione], Metribuzin [active ingredient 4-amino-6(1,1-dimethyl)-3-(methylthio)-1,2,4-triazin-5(4H)] and Chlorsulfuron [active ingredient 2-chloro-N-[(4-methoxy-6-methyl-1,3,5-triazin-2-yl) aminocarbonyl]-benzenesulfonamide] based on research trials in Canada (Moyer et al. 1990). Post emergence herbicides such as MCPA [active ingredient 4-(4-Chloro-2-methyl-phenoxy) acetic acid] and MCPB [active ingredient 4-(4-Chloro-2-methyl-phenoxy) butyric acid] can be used to control broad-leaved weeds in sainfoin fields. There are ten registered herbicides for weed control in sainfoin in Western Canada (Government of Alberta Bulletin 2014) (Table 2.1).

Sainfoin is relatively tolerant to glyphosate application. Based on the dose estimated to cause 50% mortality (LD_{50}), sainfoin seedlings are over six times, and mature plants are over 20 times, more tolerant to glyphosate than alfalfa. The application of glyphosate also has a 10-fold greater negative impact on alfalfa DM yield than it does on sainfoin (Peel et al. 2013).

Moyer (1985) used barley (*Hordeum vulgare* L.) as a companion crop with sainfoin to control weed infestation in the establishment year, however, the inclusion of barley reduced sainfoin DM yield and stand establishment. Koivisto and Lane (2001) suggested using a non-competitive grass as a companion crop to aid in weed control in the establishment year. In a European study, Dimitrova (2010) found that chemical weed control in pure stands of sainfoin resulted in higher seed yield than stands with barley as a companion crop.

Table 2.1 List of registered herbicides for sainfoin (*Onobrychis viciifolia* Scop.) in Western Canada

Herbicide name	Active ingredient
Assure II	1-quizalofop
Yuma GL	1-quizalofop
Treflan EC	3-trifluralin
Bonanza 480 EC	3-trifluralin
Rival EC	3-trifluralin
Basagran	6-bentazon
Liquid Achieve SC	1-tralkoxydim
Bison	1-tralkoxydim
Marengo	1-tralkoxydim
Poast Ultra	1-sethoxydim

Source: Government of Alberta Bulletin 2014.

2.6 Dry Matter Yield

Sainfoin can be seeded as a monoculture or in a mixture with other perennial grasses or alfalfa. Sainfoin produces approximately 5–20% less DM yield than alfalfa in Western Canada (Goplen et al. 1991). Depending upon the growing conditions, DM yield of sainfoin varied from 7–15 t ha^{-1} in Western Canada (Goplen et al. 1991). In a five-year field study in Alberta, Canada, sainfoin produced DM yield of more than 6 t ha^{-1} under rainfed conditions (Government of Alberta Bulletin 2014).

Sainfoin is more compatible with caespitose grasses in mixtures than with aggressive rhizomatous grass species. In Lethbridge, Canada, the five year average yield of sainfoin-Russian wildrye (*Psathyrostachys junceus* Fisch.) mixtures was 5.8 t DM ha^{-1}, while sainfoin-crested wheatgrass [*Agropyron cristatum* (L.) Gaertn.] and sainfoin-pubescent wheatgrass [*Thinopyrum intermedium* subsp. *Barbulatum* (Schur) Barkw. & D.R. Dewey] mixtures produced 5.2 t DM ha^{-1} and 4.8 t DM ha^{-1}, respectively (Goplen et al. 1991). In addition, sainfoin contributed 61% and 48% of total DM yield when it was grown in mixtures with Russian wildrye grass and crested wheatgrass, respectively.

In Europe, Turk et al. (2011) reported DM yields from 5.1 t ha^{-1} at the beginning of flowering to 6.5 t ha^{-1} at seed filling stages. Sainfoin has been sown with non-aggressive grasses such as meadow fescue *(Festuca pratensis* Huds.) and timothy *(Phleum pratense* L.) (Frame et al. 1998). Liu et al. (2006) reported higher forage yield, at 9.1 t DM ha^{-1}, for a sainfoin and meadow fescue mixture seeded in a ratio of 2:1 than sainfoin monoculture, which yielded 7.5 t DM ha^{-1}, based on a three-year study in the United Kingdom, ternary mixtures of sainfoin with bromegrass and crested wheatgrass yielded 8.4 t DM ha^{-1}; with bromegrass and intermediate wheatgrass 7.2 t DM ha^{-1} ; and with crested wheatgrass and intermediate wheatgrass 7.6 t DM ha^{-1} in Turkey (Albayrak et al. 2011).

2.7 Nutritive Value

Forage nutritive value of sainfoin is comparable to alfalfa and other forage legumes. Nutritive value of sainfoin is determined by cultivar, growth stage, and growing environment. A number of studies quantified the nutritive value (mainly crude protein (CP), neutral detergent fiber (NDF) and acid detergent fiber (ADF) concentrations) of sainfoin at different growth stages (Table 2.2). Nutritive value of sainfoin varied not only among the growth stages, it also varied within the same growth stages under different experimental conditions (Table 2.2). Overall, CP concentrations at the vegetative stage ranged from 195–198 g kg^{-1}, 114–177 g kg^{-1} at the flowering stage, and 130–171 g kg^{-1}at the seed filling stage. Neutral detergent fiber concentrations were in the range of 378–461 g kg^{-1} at the vegetative stage, 372–493 g kg^{-1} at the flowering stage, and 446–557 g kg^{-1}, at the seed filling stage. Acid detergent fiber concentration ranged from 286–334 g kg^{-1} at the vegetative stage, 313–433 g kg^{-1} at the flowering stage and 338–402 g kg^{-1} at the seed filling stage. After 42 days of regrowth, the nutritive value of sainfoin was similar to the first growth vegetative stage, with CP ranging from 148–186 g kg^{-1}, and NDF and ADF concentration ranging 365–454 g kg^{-1} and 337–397 g kg^{-1}, respectively.

Table 2.2 Nutritive value of sainfoin (*Onobrychis viciifolia* Scop.) at different growth stages

Growth stage	CP[a] (g kg^{-1})	NDF[b] (g kg^{-1})	ADF[c] (g kg^{-1})	References
Vegetative	195–198	378–461	286–334	Bal et al. 2006; Turk et al. 2011
Flowering	116–143	372–457	368–392	Parker and Moss 1981
Flowering	121	478	433	Khalilvandi-Behroozyar et al. 2010
Flowering	114–177	433–476	343–433	Kaplan 2011
Flowering	145	493	372	Bal et al. 2006
Flowering	125–161	—	313–371	McMahon et al. 1999
Seed Filling	130	557	402	Bal et al. 2006
Seed Filling	171	446	338	Turk et al. 2011
Regrowth (42 days)	148–186	365–454	337–397	Azuhnwi et al. 2012

[a]CP, Crude Protein; [b]NDF, Neutral Detergent Fiber; [c]ADF, Acid Detergent Fiber.

2.8 Forage Digestibility and Palatability

At a similar stage of maturity, Ditterline and Cooper (1975) found that sainfoin contained lower CP, crude fiber, and ash concentrations than alfalfa. The voluntary intake of sainfoin by sheep was 29% higher than alfalfa and pubescent wheatgrass (Karnezos et al. 1994). Sainfoin was found to be equal or more palatable to grazing animals than alfalfa (Parker and Moss 1981, Khalilvandi-Behroozyar et al. 2010). Baumont (1996) defines palatability as physical or chemical characteristics of the feed that invokes appetite. Palatability index of sainfoin was 2.7 times higher than alfalfa when evaluated with ewes (Khalilvandi-Behroozyar et al. 2010). Parker and Moss (1981) reported higher palatability for sainfoin than alfalfa when studied with heifers. In a European study, Scharenberg et al. (2007) described sainfoin as a more promising forage legume than birdsfoot trefoil (*Lotus corniculatus* L.) and chicory (*Cichorium intybus* L.) due to its high palatability and high content of metabolizable energy.

Furthermore, sainfoin has hollow stems, which are more digestible to ruminants than alfalfa (Terry and Tilley 1964). Average daily gains of 0.96 kg and 0.91 kg were reported for heifers finished on sainfoin and alfalfa-grass hay, respectively (Parker and Moss 1981). Marten et al. (1987) reported an average daily gain of 0.80 and 0.67 kg for heifers grazed on sainfoin and alfalfa pastures, respectively.

Sainfoin has a low initial rate of digestion in comparison to alfalfa which in part explains its bloat safe characteristic (Coulman et al. 2000). Sainfoin contains moderate concentrations of condensed tannins, which are polyphenolic compounds expressed in a wide range of forbs including legumes (Wang et al. 2015). Condensed tannins present in temperate forages can reduce protein loss and increase amino acid absorption by ruminants (Min et al. 2003). Tannins also play anti-microbial roles such as reducing *E. coli* in the rumen (Hassanpour et al. 2011). Recent studies showed that incorporation of sainfoin in alfalfa pastures can reduce the risk of pasture bloat in ruminants (McMahon et al. 1999; Wang et al. 2006; Sottie et al. 2014). However, most sainfoin cultivars do not persist in alfalfa stands and in new mixed stands do not regrow at the same rate as alfalfa after cutting or grazing (Jefferson et al. 1994; Acharya et al. 2013). A new sainfoin cultivar, AAC Mountainview, was developed in Canada in 2013, which showed an improved persistence in mixed stands with alfalfa and improved regrowth after grazing (Acharya 2015).

2.9 Genetic Diversity and Cultivar Development

Diversity in plant genetic resources provides an opportunity for plant breeders to develop new and improved cultivars with desirable characteristics. Understanding genetic diversity aids in classification and identification of plant accessions (Majidi et al. 2009). Inter simple sequence repeat (ISSR), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and single nucleotide polymorphisms (SNP) are molecular techniques used to identify genetic diversity in plant diversity studies.

In sainfoin, RAPD and ISSR markers were used to understand genetic diversity and relationships of regional to large worldwide collections (Table 2.3). A high within-population genetic variation ranging found 78–89% was found in various diversity studies (Table 2.3). The percentage of polymorphic bands were in the range of 66–96% (Table 2.3). The results of the sainfoin diversity studies revealed a higher level of within-population genetic diversity than among the populations (Table 2.3). A high level of within-population genetic diversity was also reported for perennial, out-crossing grasses (Fu et al. 2005; Biligetu et al. 2013). In addition, Demdoum et al. (2012) found that the genetic diversity of sainfoin accessions were associated with their geographic locations with British accessions being separate from other European accessions. Delgado et al. (2008) observed high agronomic variability among the 44 accessions based on morphological traits and was able to differentiate sainfoin germplasm based on the percentage of flowering plants in the year of seeding and the speed of regrowth after the spring cut. Mohajer et al. (2013) found high diversity among 12 Iranian accessions based on agro-morphological and quality traits of sainfoin. Availability of genetic variation is the source of genetic improvement in breeding programs. Production of synthetic varieties with improvements in agronomic and quality traits is possible by crossing genetically diverse accessions (Hosainianejad et al. 2011).

Table 2.3 Genetic diversity of sainfoin (*Onobrychis viciifolia* Scop.) assessed by different molecular markers

Location of Accession Collection	Number of accessions	Molecular Marker Type	Number of primers	Polymorphic bands	Within-population variability	Among-population variability	References
East Azerbaijan, Iran	5	RAPD[a]	5	67–85 %	89%	11%	Nosrati et al. 2012
Worldwide collection	80	ISSR[b]	22	88%	80%	20%	Zarrabian et al. 2013
Iran	36	RAPD	5	66%	78%	22%	Rasouli et al. 2013
Iran	10	RAPD	10	84%	84%	16%	Hejrankesh et al. 2014
Worldwide collection	102	ISSR	22	97%	—	—	Zarrabian and Majidi 2015

[a]RAPD, Random Amplified Polymorphic DNA; [b]ISSR, Inter Simple Sequence Repeat.

The majority of cultivated sainfoin accessions are tetraploid (2n=4x=28), while diploid (*2n*=*2x*=14) types are also found (Frame et al. 1998; Zarrabian et al. 2013). In the past decade, significant genetic improvements have been made through plant breeding in other perennial legumes such as alfalfa (Demdoum et al. 2012; Zarrabian et al. 2013). Although it was introduced to the North America in 1786, sainfoin genetic improvement has been relatively slow. The registered cultivars of sainfoin in North America are described in Table 2.4, with only three cultivars having been released in Canada. Sainfoin breeding has focused on the improvement of forage DM yield, winter survival, stand persistence and grazing tolerance. There are also a relatively large number of cultivars registered in Europe and New Zealand, including Zeus and Vala (Italy), Perly (Switzerland), Fakir (France), Emyr (Hungary) (Koivisto and Lane 2001) and G35 (New Zealand) (Rumball and Claydon 2005). Landraces such as ‘Cotswold’ and ‘Hampshire’ are also seeded in Europe.

Table 2.4 List of registered sainfoin (*Onobrychis viciifolia* Scop.) cultivars in North America

Cultivar	Released year	Country	Remarks	References
Eski	1964	USA	Dry land pasture	(USDA 2015)
Melrose	1969	Canada	Dry land hay or pasture and irrigated hay	(Cooke et al. 1971)
Remont	1971	USA	Multiple-cut hay harvest and pasture	(USDA 2015)
Renumex	1979	USA	Rapid regrowth	(USDA 2015)
Nova	1980	Canada	High dry matter yield and winter hardiness	(Hanna 1980)
Shoshone	2006	USA	High tolerant to northern root-knot nematode	(Gray et al. 2006)
Delaney	2007	USA	Multiple cut system	(USDA 2015)
AAC Mountainview	2013	Canada	Rapid regrowth, persistent in alfalfa-sainfoin mixture	(Acharya 2015)

Chapter 3. Genetic diversity and relationship of sainfoin (*Onobrychis viciifolia* Scop.) germplasm as revealed by amplified fragment length polymorphism markers

3.1 Abstract

Sainfoin (*Onobrychis viciifolia* Scop.) is a perennial forage legume, widely distributed in the northern temperate regions of the world. However, its genetic improvement has been relatively slow, in part due to the lack of informative molecular characterization of sainfoin germplasm. An attempt was made to evaluate genetic diversity and relationships among 38 sainfoin accessions collected from different regions of the world using amplified fragment length polymorphism (AFLP) markers. Five AFLP primer pairs were used to assess 367 individual plants, which produced 1,042 polymorphic AFLP bands. The frequencies of the scored bands in all assayed individuals ranged from 0.003 to 0.973 with a mean value of 0.165. The analysis of molecular variance (AMOVA) revealed higher within-population (84.1%) genetic variation than among populations (15.0%) or among groups (0.9%). The genetic distance calculated based on inter-population distance matrices was significant for most accessions, but it was not significant for sister breeding lines, or accessions sharing similar parents. A dendrogram of the collected accessions showed two clusters at an inter-population genetic distance coefficient of 0.36. The information collected on the sainfoin accessions is significant for understanding sainfoin genetic diversity and will be useful for current sainfoin breeding efforts.

3.2 Introduction

Sainfoin (*Onobrychis viciifolia* Scop.) belongs to the Fabaceae family and Hedysareae tribe, and is native to south central Asia (Burton and Curley 1968). The countries of Iran and Turkey are considered to be the main centers of diversity (Yildiz et al. 1999). Sainfoin adapts well to a wide range of climatic and soil conditions (Frame 2005). It is an open-pollinated species with both tetraploid ($2n=4x=28$) and diploid ($2n=2x=14$) forms (Frame et al. 1998; Zarrabian et al. 2013). Sainfoin has been cultivated for forage production, but it produces approximately 5–20% less dry matter yield than alfalfa (*Medicago sativa* L.) in Western Canada (Goplen et al. 1991). It displays several challenges for genetic improvement including poor regrowth, lack of persistence in forage mixtures, low tolerance to waterlogging and frost, and its poor competitive ability in the early stages of development (Hayot-Carbonero 2011; Bhattarai et al. 2016; Kempf et al. 2016).

Although sainfoin was introduced to North America in 1786 (Hanna 1972), its forage production on this continent largely relies on a few cultivars. In Canada, Melrose (released in 1969), Nova (released in 1980) and AAC Mountainview (released in 2013) are the only three available cultivars of sainfoin. In recent years, there has been increasing demand for high yielding sainfoin cultivars with good stand persistence and rapid recovery under grazing in temperate regions of North America. However, the genetic improvement of sainfoin is relatively slow, in part due to the lack of informative molecular characterization of sainfoin germplasm. Sainfoin breeding would be more effective with a better understanding of genetic diversity and relationships of diverse sainfoin germplasm (Majidi et al. 2009). The maintenance of high genetic diversity is also crucial for sainfoin breeding, as it is highly susceptible to inbreeding depression (Rasouli et al. 2013).

Morphological, anatomical and molecular characteristics have been employed to study genetic diversity. However, the inference of genetic diversity using molecular markers should be more informative than morphological traits as the former is not affected by environmental factors (Awasthi et al. 2004). DNA-based fingerprinting technologies have been applied in genetic studies in a wide range of plant species. In sainfoin, random amplified polymorphic DNA (RAPD; Williams et al. 1990) and inter-simple sequence repeats (ISSR) markers have been utilized to analyze its genetic diversity (Nosrati et al. 2012; Rasouli et al. 2013; Zarrabian et al. 2013; Avci et al. 2014; Hejrankesh et al. 2014; Zarrabian and Majidi 2015). Efforts have also been made to develop amplified fragment length polymorphisms (AFLP; Vos et al. 1995) and simple sequence repeat (SSR) markers with little success (Hayot-Carbonero 2011; Demdoum et al. 2012) until the recent discovery of 101 informative SSR markers (Kempf et al. 2016). More recently, an RNA-seq analysis revealed 3,786 potential SSRs and 77,000 putative single nucleotide polymorphisms (SNPs) (Mora-Ortiz et al. 2016), opening new opportunities to characterize and study sainfoin germplasm for breeding and research.

This study undertook an AFLP analysis of sainfoin germplasm to assess the genetic diversity and genetic relationships among 38 sainfoin accessions collected from different regions of the world. AFLP markers were selected because they are the most widely used markers for detecting genetic variation in cross-pollinated and polyploid crops with little genomic information and they do not require any prior sequence information (Williams et al. 1990; Vos et al. 1995). The AFLP technique is also efficient for detecting polymorphisms, being more informative than RAPD and ISSR (Powell et al. 1996), and allows for effective genotyping by rapidly generating hundreds of highly reproducible DNA markers (Loh et al. 2000). AFLP-based genetic diversity analyses have been reported in many forage crops, such as meadow bromegrass

(*Bromus riparius* Rehmann) (Ferdinandez and Coulman 2002), alfalfa (Segovia-Lerma et al. 2003), perennial ryegrass (*Lolium perenne* L.) (Skot et al. 2002; Jonaviciene et al. 2014), blue grama (*Bouteloua gracilis* (Willd. Ex Kunth) Lag. Ex Griffiths) (Fu et al. 2004a), little bluestem (*Schizachyrium scoparium* (Michx.) Nash) (Fu et al. 2004b), wild orchardgrass (*Dactylis glomerata* L.) (Peng et al. 2008), napiergrass (*Pennisetum purpureum* Schum.) (Harris et al. 2010), side-oat grama (*Bouteloua curtipendula* (Michx.) Torr.) (Biligetu et al. 2013), Nuttall's salt-meadow, or alkali grass (*Puccinellia nuttalliana* (Shultes) Hitchc.) (Liu et al. 2013), Kenyan (*Themeda triandra* Forssk.) (Dell'acqua et al. 2014), and *Brachypodium* species (Zhang et al. 2012).

3.3 Materials and Methods

3.3.1 Plant materials

The thirty-eight accessions of sainfoin used for this study are shown in Table 3.1. Seedlings were grown in the greenhouse for eight weeks prior to being transferred to the field in July 2014 near Saskatoon, SK, Canada (52° 07' N, 106° 38' W). Thirty-two individual plants for each accession were space planted in the field using a randomized complete block design with four replications consisting of eight individual plants per replication. The spacing was 1 m between any two individual plants.

Table 3.1 Information for the 38 sainfoin accessions studied by AFLP analysis

Code	Accession	Country of Origin	Continent
1	PI636518	China	Asia
2	PI258773	USSR	Asia
3	Marinella	Slovakia	Europe
4	Visnovsky	Czech republic	Europe
5	PI319062	Spain	Europe
6	Dnepropetrovsk	USSR	Asia
7	CN45949	China	Asia
8	PI313049	Poland	Europe
9	WY-PX2-94	United States	North America
10	Shoshone	United States	North America
11	Lupinella	Italy	Europe
12	PI318606	Switzerland	Europe
13	PI380949	Iran	Asia
14	LRC 3519	Canada	North America
15	LRC 3901	Canada	North America
16	Renumex	United States	North America
17	CN38745	Turkey	Asia
18	PI338651	Morocco	Africa
19	Remont	United States	North America
20	PI440577	Kazakhstan	Asia
21	Melrose	Canada	North America
22	PI568208	Turkey	Asia
23	Hampshire Common	England	Europe
24	PI110397	USSR	Asia
25	Svkpiestt	Slovakia	Europe
26	PI313066	Bulgaria	Europe
27	SL895	Canada	North America
28	PI313056	Norway	Europe
29	Giant	England	Europe
30	PI502554	USSR	Asia
31	PI577670	China	Asia
32	PI110404	USSR	Asia
33	Nova	Canada	North America
34	CN31800	France	Europe
35	PI273791	Ukraine	Europe
36	SCO38401	Canada	North America
37	SF-Laramie-73	United States	North America
38	PI494667	Romania	Europe

3.3.2 Tissue collection and DNA extraction

In August 2015, fresh leaves of 10 randomly selected plants were sampled from each of the 34 sainfoin accessions in the field plot. Due to a low winter survival rate, fresh leaves of six randomly selected individual plants were sampled for the accessions PI568208, CN45949 and LRC3519, and nine individual plants for PI380949. Leaf samples were kept in a cooler on ice during transportation from the field to lab. The leaf samples were freeze-dried in a Labconco Freeze Dry System for 48 h and stored at −20°C. Approximately 10–15 mg of leaves from each plant were placed in a 1.5 mL micro-centrifuge tube with three, 2 mm glass beads. The leaf tissue was ground to a fine powder using a tissue lyser. The DNA was extracted using a DNeasyTM Plant Mini Kit (Qiagen Inc. Mississauga, ON) according to the manufacturer's instructions. Extracted DNA was quantified using a Nanodrop ND-8000 (Thermo Fisher Scientific, Wilmington, DE, USA), followed by dilution to 25 ng μL^{-1} for AFLP analysis.

3.3.3 AFLP analysis

Amplified fragment length polymorphism analysis was performed using the AFLP™ Analysis System 1 (Life Technologies Burlington, ON) according to Vos et al. (1995) with some modifications. DNA was digested with enzymes *Eco*RI and *Mse*I at 37°C for 2 h and inactivated at 70°C for 15 min. Immediately after restricted digestion of genomic DNA, adapters were ligated to the restriction fragments with T4 DNA ligase at room temperature (20–25°C) for 2 h to create primary templates. The ligated DNA was diluted 10 fold in the 96 well plate with 0.1M TE (10mmol l^{-1} Tris-HCl pH 8.0, 0.1mmol l^{-1} ETDA, distilled water), then stored at −20°C. The ligated DNA was pre-amplified with pre-amplification primers. The amplified product was diluted 50 fold with 0.1M TE and used for selective amplification. Selective amplification reactions were performed using pre-amplified DNA and fluorescently labelled FAM *Eco*RI primers and unlabeled *Mse*I primers. Ten *Eco*RI: *Mse*I primer pairs were initially screened on six leaf samples and the five most informative primer combinations were selected and used for selective amplification. PCR products were diluted 40 fold with ultra-pure water. One microliter of diluted sample was combined with 8.75 µl of HI-DYE formamide and 0.25 µl of 600 LIZ and denatured for 3 minutes at 95°C and analyzed on an Applied Biosystems 3100x1 genetic analyzer (Applied Biosystems Life Technologies, Burlington, ON). Data were collected using GeneMapper software version 4.1 (Applied Biosystems Inc.) with bin size set at 1 bp and scored from 60 to 600 bp. The minimal acceptable peak height was 50 units. As a check, two randomly selected individuals were repeated on each of the plates to minimize technical and scoring errors.

3.4 Data analysis

Data from GeneMapper were exported to Microsoft Excel and converted into a binary matrix scoring as 1 (present) or 0 (absent) with missing values recorded as 9. The analysis of AFLP polymorphisms was carried out by counting the number of polymorphic bands and generating summary statistics. The polymorphism information content (PIC) for each primer was calculated to measure the informativeness of a marker by using the following formula:

$PIC_i = 2 f_i (1-f_i)$ (Roldan-Ruiz et al. 2000),

where 'i' is the ith primer, PIC_i is the polymorphism information content of marker 'i', f_i is the frequency of the amplified allele and $(1-f_i)$ is frequency of the null allele.

To assess AFLP variation, Arlequin software version 3.05 (Excoffier et al. 2005) was used to perform an analysis of molecular variance (AMOVA). The genetic associations of 38 sainfoin accessions were analyzed using NTSYS-pc 2.1 (Rohlf 1997) based on the inter-population distance matrices of the Phi statistic obtained from the AMOVA. A dendrogram of the 38 sainfoin accessions was created using NTSYS-pc 2.1 using the AFLP-based unweighted pair group method with arithmetic mean (UPGMA). The genetic association of 367 individual plants was further assessed without restriction to population origin. A neighbor-joining (NJ) analysis was made using PAUP* (Swofford 1998) based on the original dataset of 1,042 alleles, and radiation tree was displayed using MEGA 3.01 (Kumar et al. 2004). Principal coordinates analysis (PCoA) was done using NTSYS-pc 2.1 with the Jaccard coefficient.

3.5 Results

3.5.1 AFLP markers

The five *Eco*RI: *Mse*I-based primer pairs yielded clear and informative banding patterns useful for genetic analysis of sainfoin. The observed band frequencies ranged from 0.003 to 0.973 with a mean value of 0.165 (Table 3.2). The mean value, minimum and maximum band frequencies for each primer pair are shown in Table 3.2. The lowest mean frequency (0.126) was observed for the primer pair E+ACC/M+CTC while the highest mean frequency (0.196) was observed for the primer pair E+ACA/M+CAT. The PIC value ranged from 0.129 to 0.186 with a mean value of 0.167 (Table 3.2).

Table 3.2 Polymorphisms generated among 367 sainfoin plants by five AFLP primer pairs

Primer pairs	AFLP bands scored	PIC[a]	Frequency of scored bands		
			Mean	Maximum	Minimum
E+AAG/M+CAC	222	0.186	0.186	0.978	0.003
E+ACA/M+CAG	261	0.167	0.165	0.970	0.003
E+ACA/M+CAT	244	0.180	0.196	0.973	0.003
E+ACC/M+CTC	166	0.129	0.126	0.967	0.003
E+AGC/M+CAC	149	0.169	0.151	0.976	0.003
All	1042	0.167	0.165	0.973	0.003

[a]Polymorphism information content.

3.5.2 Genetic variation

The 38 accessions were categorized into four groups based on geographic origin (Table 3.1). The majority of genetic variability occurred within accessions (84.1%) with less observed among accessions (15.0%) or among groups (0.9%) (Table 3.3). Although variation among the four groups made up a small fraction of the total genetic variation, it was significant (*P=0.013*) based on the random permutation test. Within-accessions variation analyzed for each group was 93%, 85%, 77%, and 72% for accessions from North America, Europe, East Asia and West Asia, respectively (Table 3.3). Based on the percentage of polymorphic loci, the five most diverse accessions were WY-PX2-94 (United States), Lupinella (Italy), LRC3901 (Canada), PI568208 (Turkey), and CN31800 (France), respectively. The five least diverse accessions were CN38745 (Turkey), Marinella (Slovakia), LRC3519 (Canada), CN45949 (China), and PI636518 (China), respectively (data not shown).

Table 3.3 Analysis of molecular variance (AMOVA) based on AFLP data generated among 38 sainfoin accessions collected from 20 countries originating from four geographic regions (North America, Europe, East Asia, and West Asia)

Source of variations		Degrees of freedom	Sum of squares	Variance component	Distribution of variation (%)	*P-value*
All	Among Groups	3	778.9	0.76	0.90	*0.013*
	Among Accessions	34	6641.9	12.80	15.03	*<0.001*
	Within Accession	329	23569.7	71.64	84.07	*<0.001*
	Total	366	30990.5	85.20		
North America	Among Accessions	10	1299.4	5.50	6.67	*<0.001*
	Within Accession	95	7312.6	76.97	93.30	
	Total	105	8612.0	82.47		
Europe	Among Accessions	14	2787.9	12.59	14.68	*<0.001*
	Within Accession	135	9882.9	73.20	85.32	
	Total	149	12670.8	85.75		
East Asia	Among Accessions	7	1727.0	19.21	22.88	*<0.001*
	Within Accession	68	4402.3	64.74	77.12	
	Total	75	6129.3	83.95		
West Asia	Among Accessions	3	827.5	24.54	27.84	*<0.001*
	Within Accession	31	1971.9	63.61	72.16	
	Total	34	2799.4	88.15		

3.5.3 Genetic association

Inter-population genetic distance was statistically significant between all but 14 pairs (data not shown). A dendrogram produced with the UPGMA method placed the 38 accessions into two main clusters (Figure 3.1). Cluster I (PI636518 from China) and cluster II (all other accessions). Cluster II consisted of many sub-clusters, which were aligned together by genetic background and origin. The cultivars or breeding lines derived from United States (WY-PX2-94, Shoshone and SF-Laramie-73) were grouped with accessions from USSR (PI258773), and Ukraine (PI273791). This sub-cluster was loosely grouped with the other two cultivars from the United States (Renumex and Remont). Another distinct sub-cluster in the dendrogram includes Canada’s oldest cultivar Melrose aligned closely with Canadian accession (SL895) and accessions from USSR (PI313056 and PI110404), and England (Giant). Another sub-cluster consists of Canadian cultivar Nova aligned together with Canadian accession (SCO38401), accessions from Kazakhstan (PI440577), France (CN31800) and USSR (PI502554). Accessions from Europe and USSR grouped together in a separate sub-cluster (PI319062, Dnepropetrovsk, Hampshire Common, and PI313066). There was no clear clustering of accessions from each of the four groups.

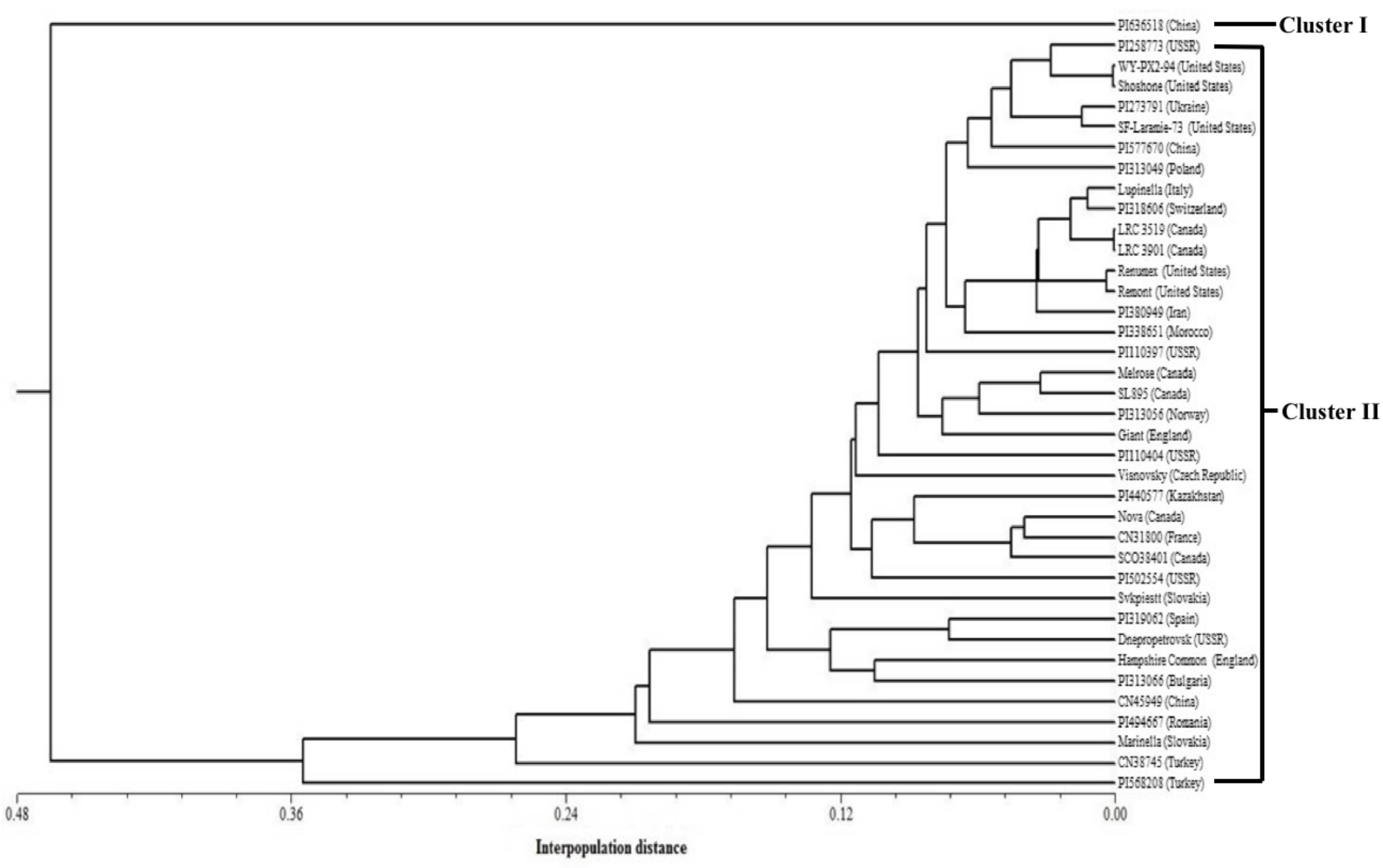

Figure 3.1 AFLP-based genetic relationships among the 38 sainfoin accessions grouped based on UPGMA cluster analysis using inter-population distance.

Phylogenetic analysis of the 367 individual plants using the NJ model with the phi-distance method is shown in Figure 3.2. Individuals from North America and Europe were distributed relatively uniformly around the NJ tree while individual plants from East or West Asia showed a tendency to cluster together. Similarly, principal coordinates analysis (PCoA) revealed a distinctive cluster for accession PI636518 (China), however, the individual plants from other accessions largely overlapped (data not shown).

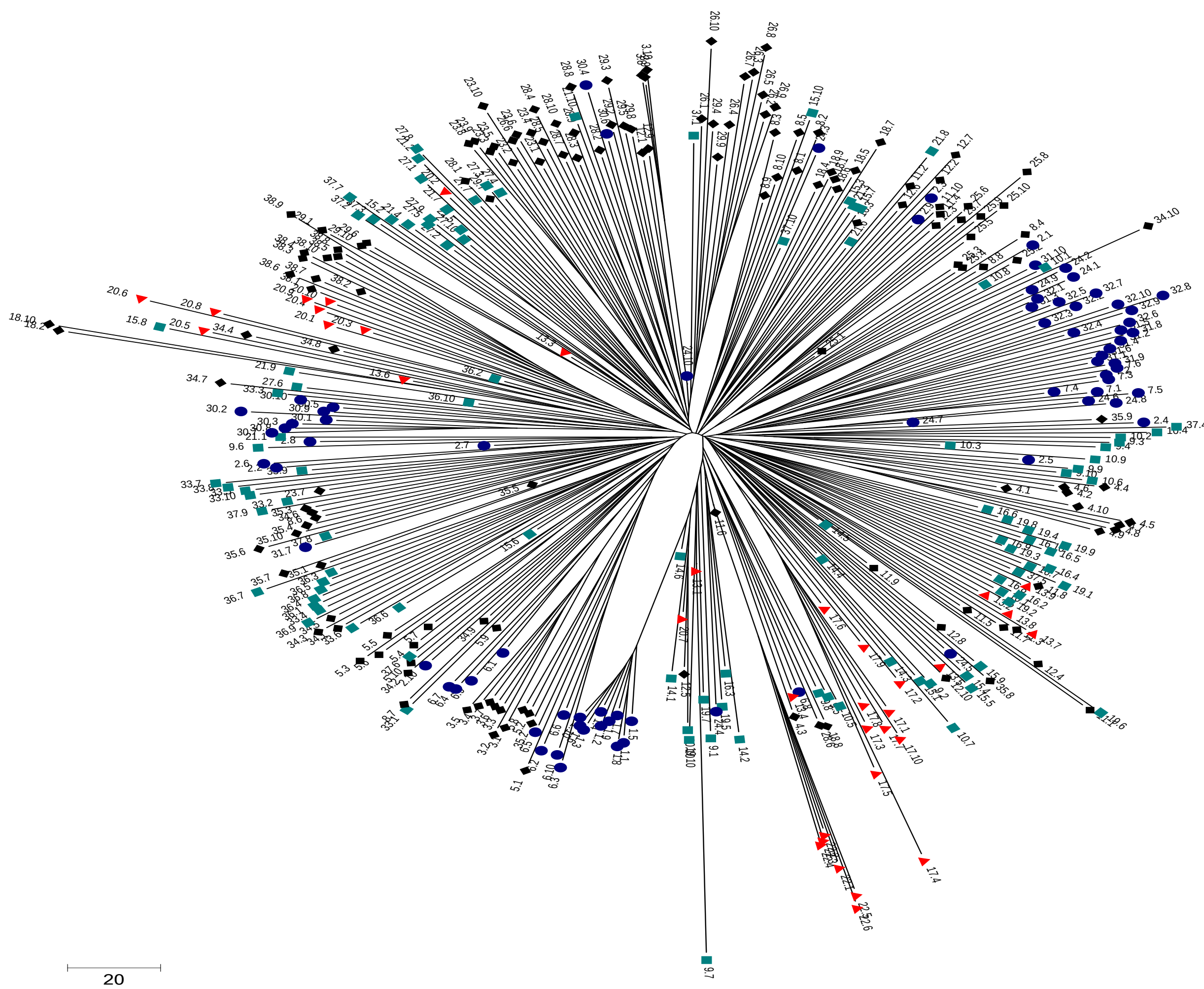

Figure 3.2 Genetic relationships among 367 sainfoin individuals as revealed by AFLP-based neighbor-joining (NJ) tree. Individuals from North America (green), Europe (black), East Asia (blue) and West Asia (red) are highlighted with different colors. Each individual plant is labeled with a code representing its accession (Table 3.1) and plant number.

3.6 Discussion

As sainfoin is a less developed forage legume with limited genetic information available, this study generated a set of useful genetic information. Five AFLP primer combinations produced clear polymorphic marker profiling of the sainfoin accessions and indicated that significant genetic variation existed within- or among- accessions and among groups. The accessions PI636518 (China) and PI568208 (Turkey) were found to be the most genetically distinct accessions. The majority of accessions were grouped together in sub-clusters based on cultivar pedigree and origin. This information is important for selecting genetically diverse parent populations for sainfoin breeding programs. Although AFLP markers are dominant in nature, and thus not able to distinguish homozygous and heterozygotes alleles, these results showed that they are an informative and suitable marker system to study genetic diversity and relationships among sainfoin genotypes in the absence of known genomic information.

This study found high within-accession genetic variation (84%), which was similar to the high within-population variation observed for wild germplasm of sainfoin (Nosrati et al. 2012) and regional (Iranian) populations of sainfoin (Rasouli et al. 2013; Zarrabian et al. 2013; Hejrankesh et al. 2014). High levels of within-population variation based on AFLPs was also reported for other out-crossing perennial forage species such as perennial ryegrass (Guthridge et al. 2001), white clover (*Trifolium repens* L.) (Kolliker et al. 2001), fringed brome (*Bromus ciliates* L.) (Fu et al. 2005), plains rough fescue [*Festuca hallii* (Vasey) Piper.] (Qui et al. 2007), and side-oat grama (Biligetu et al. 2013). Hamrick and Godt (1989) showed that out-crossing plant species tend to exhibit high within-population (80–90%) genetic variation. Sainfoin is an insect pollinated perennial forage legume, which is distributed worldwide from sub-tropical to temperate regions of the world. This study confirmed the high percentage of outcrossing expected in this species, in contradiction to the recent finding of up to 65% self-fertilization in an artificially directed pollination population (Kempf et al. 2015). Also, this study revealed higher within-population genetic variation for the accessions from the North America (93%) and Europe (85%) than the accessions from East Asia (77%) or West Asia (72%). Further studies with large sample sizes are needed to clarify the discrepancy.

PI636518 from China and PI568208 from Turkey were the most diverse lines based on UPGMA analysis. This finding is consistent with the phenotypic characteristics observed in this field study. Plants from accession PI636518 have short stems, small seeds, and low forage biomass production. Accession PI568208 had glossy leaves and a long spiked seed pod. The composition of many sub-clusters in the dendrogram could be explained in part by their pedigrees and origins. For example, the majority of the accessions from the United States clustered closely together reflecting their common ancestry. The cultivar Renumex was developed from Remont (USDA 2015) and WY-PX2-94 and Shoshone are also genetically related (Gray 2004). Similarly, accessions LRC3519 and LRC3901, which are sister breeding lines from Lethbridge, Canada showed a non-significant difference in genetic distance. In contrast, the accessions from England (Giant and Hampshire Common) were clustered distinctly apart. This was likely because the cultivar Giant was developed from accessions from the Middle East, which was clustered with accessions from USSR (PI313056 and PI110404) (Hayot-Carbonero et al. 2011). On the other hand, Hampshire Common originated from central Europe, and clustered closely with accessions from Bulgaria (PI313066). The Canadian accessions (Nova and SCO38401) were clustered together with an accession from Kazakhstan (PI440577) supporting the fact that Nova originated from Kazakhstan (Hanna 1980). The Canadian sainfoin cultivar Melrose was developed from USSR lines (Hanna et al. 1970) and Melrose clustered with

USSR accessions (PI110397 and PI110404). Thus, the results obtained on genetic relationships are informative for the development of synthetic sainfoin cultivars. Future sainfoin breeding could potentially achieve more heterosis by crossing with genetically distinct accessions such as PI636518 (China), PI568208 (Turkey), CN38745 (Turkey), Marinella (Slovakia) and PI494667 (Romania).

Chapter 4. Characterization of phenotypic and nutritional traits of sainfoin (*Onobrychis viciifolia* Scop.) germplasm

4.1 Abstract

This study was carried out to compare agro-morphological traits and nutritive value of 38 sainfoin (*Onobrychis viciifolia* Scop.) accessions grown in Western Canada. Field plots was established in July 2014 using a randomized complete block design with four replications near Saskatoon, SK, Canada. Data were collected in 2015 and 2016. Analysis of variance (ANOVA) revealed significant differences among sainfoin accessions for all of the measured traits. The accessions showed a wide range of variation for forage DM yield, which ranged from 74–239 g plant^{-1}, plant height, ranging from 37–70 cm, and seed yield that ranged from 5–64 g plant^{-1}. The accessions from North America, such as Melrose, SCO38401, Shoshone and WY-PX2-94, had the highest forage DM yield among the 38 accessions. Forage DM yield was positively correlated with plant height (r=0.82, $P<0.001$), spring vigor (r=0.43, $P<0.001$), stem number (r=0.75, $P<0.001$), 1000-seed weight (r=0.29, $P<0.001$), growth rate (r=0.72, $P<0.001$), regrowth (r=0.30, $P<0.001$), NDF (r=0.71, $P<0.001$) and ADF (r=0.74, $P<0.001$). Forage DM yield had a negative correlation with days-to-flower (r=−0.57, $P<0.001$) and CP concentration (r=−0.62, $P<0.001$). Cluster analysis grouped 31 sainfoin accessions into three main groups according to the agro-morphological and nutritive value. Several promising accessions were identified, which can serve as source populations to develop cultivars with high forage yield, nutritive value, and winter survival.

4.2 Introduction

Sainfoin (*Onobrychis viciifolia* Scop.) is an important perennial forage legume which is widely distributed in northern temperate regions of the world. It is a widely used forage legume because of its adaptability, palatability, high nutritional value and non-bloating characteristics (Goplen et al. 1991; Frame 2005; Delgado et al. 2008). In North America and Europe, sainfoin is generally grown as a monoculture or in mixtures with perennial grasses or alfalfa (*Medicago sativa* L.) (Goplen et al. 1991; Frame et al. 1998; Hayot-Carbonero et al. 2011). In recent years, sainfoin has been seeded with alfalfa to reduce bloat incidents in grazing animals due to its high condensed tannin (Sottie et al. 2014). Sainfoin is resistant to alfalfa weevil (*Hypera postica* Gyll.) (Hanna et al. 1972; Ditterline and Cooper 1975), thus, it can replace alfalfa in areas with high infestation of alfalfa weevil.

Characterizing agro-morphological traits is crucial to plant germplasm evaluation in a breeding program because it not only provides baseline phenotypic and agronomic information, but is also useful for estimation of genetic variation (Fufa et al. 2005). Hanna (1993) reported plant height reduction in forage crops generally decreases total DM yield, but increases leaf proportion and forage quality. High levels of variability among sainfoin accessions for forage quality and DM yield have been reported (Nakhjavan et al. 2011). Previous agronomic studies on sainfoin revealed high level of variation among populations for a wide range of agronomic traits (Delgado et al. 2008; Nakhjavan et al. 2011; Hayot-Carbonero 2011; Zarrabian et al. 2013; Jafari et al. 2014). The majority of these studies focused on regional or local germplasm collections in Europe or countries within the Middle East, such as Iran. Evaluation of a large number of germplasm accessions with diverse origin is an important step toward increasing genetic variation in a given environment. For example, winter survival is one of the most important traits for sainfoin intended for production in Western Canada. Evaluation of agro-morphological traits have been conducted intensively in other forage crops like alfalfa (Warbuton and Smith 1993; Smith et al. 1995; Abbasi et al. 2006 2007; Basafa and Taherian 2009), Russian wildrye [*Psathyrostachys juncea* (Fischer) Nevski] (Berdahl et al. 1999) and white clover (*Trifolium repens* L.) (Lane et al. 2000). Although sainfoin is considered an important forage legume with high hay and pasture production potential in Western Canada, information on agro-morphological and nutritional traits is lacking.

This study was conducted to evaluate agro-morphological characteristics and nutritive value of 38 sainfoin accessions originating from Europe, Asia, Africa, and North America under Western Canadian soil and growing conditions. The information will be used to identify superior genetic materials for future sainfoin breeding efforts.

4.3 Materials and methods

4.3.1 Plant materials and experimental design

Field plots were established in July 2014 at the Agriculture and Agri-Food Canada (AAFC) Saskatoon Research and Development Centre, Saskatoon, SK, Canada (52° 07’ N, 106° 38’ W) for 38 sainfoin accessions representing 20 countries (Table 3.1). The seedlings were grown for eight weeks in a greenhouse before being transplanted to the field. Thirty-two individual plants of each accession were arranged in the field in a randomized complete block design with four replications of eight individual plants in each replication. The spacing was 1m between any two individual plants. The soil was a Sutherland clay loam (Dark Brown Chernozem, Typic Haploboroll) (Acton and Ellis 1978). Weather data was obtained from Environment Canada (climate.weather.gc.ca).

4.3.2 Agro-morphological data collection

Data were collected during the 2015 and 2016 growing seasons. Agro-morphological data were collected from May to September at different developmental stages of sainfoin as described by Borreani et al. (2003). Winter survival rate (%) of an accession was calculated by dividing the number of surviving plants in the spring by the total number of plants originally planted. Spring vigor was visually scored at the late vegetative stage using a 1–5 scale (poor to good) based on spring growth, plant size, leafiness, disease, and stem density as described by Cosgrove et al. (2001). Spring vigor was scored on June 5, 2015 and May 26, 2016. Days to flowering was recorded for all surviving plants by counting the number of days from May 1 to the date the first flower emerged on each plant. Plant height of individual plants was measured twice on June 1 and June 11 in 2015, and May 26 and June 6 in 2016. Height was recorded for the tallest culm near the center of the plant, and growth rate per day was calculated from the two heights measured. The number of stems per plant was counted on June 15, 2015 and June 9, 2016, respectively, for three randomly selected individual plants per replication. Due to winter kill, forage DM yield was determined for 31 of 38 accessions using three of four replications. Two randomly selected individual plants in each replication were hand harvested to 5 cm stubble height at the late flowering stage in June 24, 2015 and June 14, 2016. Harvested plant samples were dried for 48 h at 60°C in a forced air oven for DM determination. The plants were scored for regrowth after 30 d of growth following harvest. Regrowth was visually scored on a 1–5 scale (1-poor, 5-good) based on plant height and stem density on July 23, 2015 and July 14, 2016. After harvesting for DM yield, the remaining plants in each accession were allowed to set seed and seeds were hand harvested to determine seed yield per plant. Approximately 10,000 leaf cutters bees were used for pollinating the plants each year. Seeds were threshed in mid-September of each year. An electronic seed counter (Agriculex Inc. Guelph, ON) was used to count seeds to determine 1000-seed weight.

4.3.3 Forage nutritive value

Sainfoin plants were sampled at the late flowering stage to a stubble height of 5 cm during the growing seasons of 2015 and 2016 for forage nutritive value determination. The samples were dried at 60°C for 48 h in a forced-air oven, then ground in a Wiley mill (Thomas-Wiley, Philadelphia, PA) to pass through a 1-mm mesh screen (Cyclone Mill, UDY Mfg., Fort Collins, CO). The ground samples were stored in plastic bags prior to crude protein (CP), neutral detergent fiber (NDF) and acid detergent fiber (ADF) determination. Nitrogen content was determined with the Dumas combustion method using a Thermo Scientific Flash 2000 Elemental Analyzer (Thermo Fisher Scientific, Netherland). CP was calculated as crude protein = nitrogen concentration × 0.625 × 1000. Neutral detergent fiber and ADF concentrations were analyzed using an automated Ankom^{2000} fiber analyzer (ANKOM Technology, Macedon, NY) according to the manufacturer's instructions (http://ankom.com/09_procedures/procedures.shtml). In brief, ANKOM F57 filter bags were filled with 500 mg of ground samples and heat sealed. Samples were extracted with neutral detergent or acid detergent and the residue was weighed to determine NDF and ADF. Moisture content of the samples was determined after analysis to express the nutritive value on a DM basis.

4.4 Statistical Analysis

Data were analyzed using the R software package, version 3.3.2 (R core team 2016). An analysis of variance (ANOVA) was performed using the Mixed model procedure to compare 38 sainfoin accessions for winter survival, plant height, growth rate, spring vigor, days to flower, stem number, seed yield and 1000-seed weight. ANOVA was also performed for forage DM yield, regrowth, CP, NDF, and ADF using the Mixed model procedure among 31 accessions. Accession, year and their interaction were considered as fixed effects, while replication was taken as a random effect. For each trait, if the ANOVA indicated significant differences at the $P<0.05$ level, the means were separated using the least significant difference (LSD) method.

The Euclidean distance matrix of 31 accessions using 13 agro-morphological and nutritive values was constructed using R software. Clustering of accessions using the Euclidean distance matrix was performed by SAHN in NTSYS-pc 2.1 using the unweighted pair group method with arithmetic mean (UPGMA) (Rohlf 1997). Pearson correlation coefficients were determined and principal component analysis (PCA) was conducted in SigmaPlot 13.0 software for all measured traits.

4.5 Results

4.5.1 Environmental conditions

The average monthly air temperature and total precipitation are shown in Figure 4.1. Growing season air temperature during the 3-yr study was close to the long-term mean. In the establishment year in 2014, the amount of precipitation was slightly less than the long-term average. Almost no rainfall was recorded during May and June in 2015 in Saskatoon, SK, but sainfoin produced relatively high forage DM yield and seed yield. Cumulative precipitation during the growing season of 2016 was higher than the long-term average and sainfoin plants showed vigorous growth.

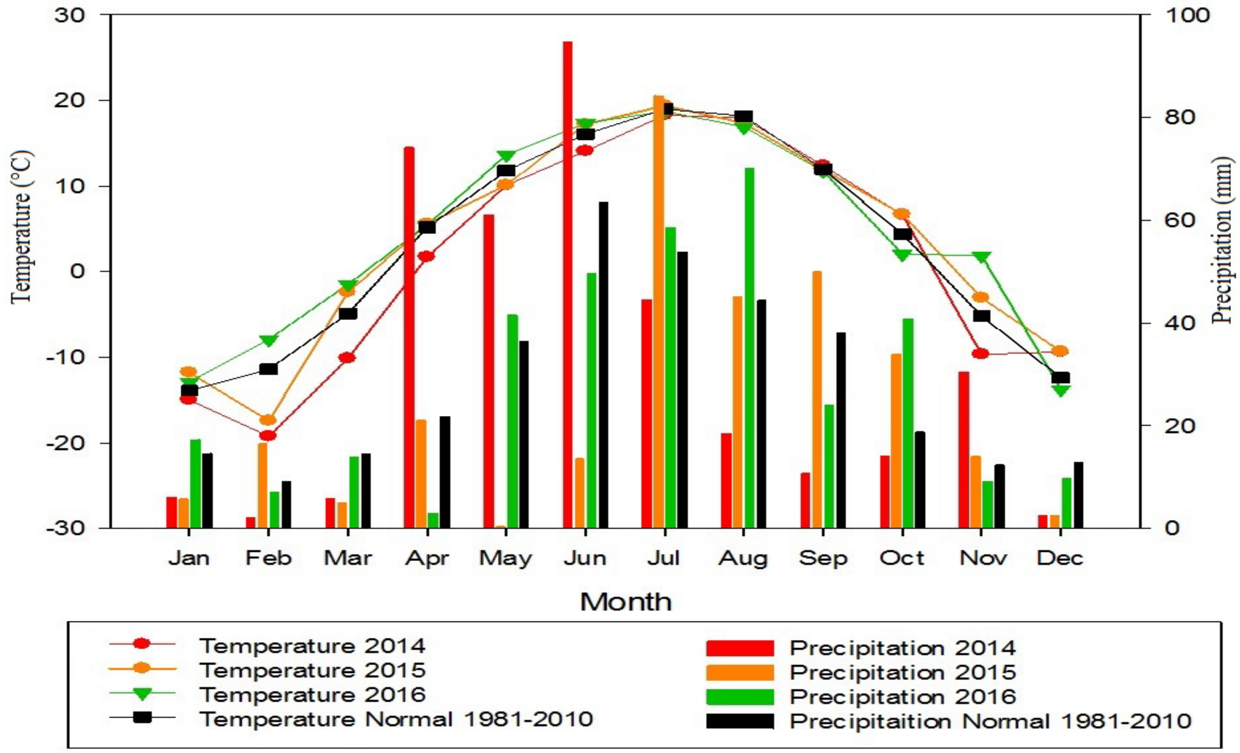

Figure 4. 1 Monthly average air temperature (°C) and total precipitation (mm) at Saskatoon, SK, Canada in 2014, 2015, 2016 and the long-term averages (1981–2010).

Table 4.1 Analysis of variance of 38 sainfoin accessions evaluated for 14 traits in 2015 and 2016 at Saskatoon, SK, Canada

Source	df[a]	WS	PH	GR	SV	DF	SN	SY	TSW	DMY	RG	CP	NDF	ADF
Accession	37	***	***	***	***	***	***	***	***	***	***	***	***	**
Year	1	ns	***	***	**	***	***	**	***	***	ns	***	***	***
Accession × Year	37	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns

WS=winter survival; PH=plant height; GR=growth rate; SV=spring vigor; DF=days to flower; SN=stem number; SY=seed yield; TSW=1000-seed weight; DMY=dry matter yield; RG=regrowth; CP=crude protein; NDF=neutral detergent fiber; ADF=acid detergent fiber.

***, significant at $P<0.001$; **, significant at $P<0.01$; *, significant at $P<0.05$; ns, not significant.

[a]Degree of freedom for Accession and Accession × Year for traits DMY, RG, CP, NDF, and ADF is 30.

4.5.2 Agro-morphological and nutritional traits

4.5.2.1 Winter survival

Winter survival was poor for 7 of 38 sainfoin accessions. The seven accessions are CN45949 (China), PI319062 (Spain), PI313066 (Bulgaria), PI380949 (Iran), PI568208 (Turkey), LRC3519 (Canada) and LRC3901 (Canada). The ANOVA revealed that winter survival was significantly different among accessions ($P<0.001$), but it was not affected by year ($P=0.07$) and accession × year interaction ($P=0.99$) (Table 4.1). Winter survival rate of sainfoin accessions ranged from 20% (LRC3519) to 94% (PI494667). The five accessions with high winter survival were PI494667 (Romania), SCO38401 (Canada), Nova (Canada), Melrose (Canada) and Shoshone (United States).

4.5.2.2 Forage DM yield

Forage DM yield was significantly different among accessions ($P<0.001$). Forage DM yield was significantly ($P<0.001$) higher in 2016 than in 2015, but all accessions were ranked similarly in both years ($P=0.07$) (Table 4.1). Averaged across the two years, the forage DM yield ranged from 74 g plant^{-1} (PI636518) to 239 g plant^{-1} (WY-PX2-94), with an average of 164 g plant^{-1} (Figure 4.2). The five accessions with the highest DM yield were WY-PX2-94 (United States), Shoshone (United States), SCO38401 (Canada), Melrose (Canada) and PI258773 (USSR). In general, the cultivars originating from North America produced high forage DM yields at Saskatoon, Canada.

4.5.2.3 Regrowth

Regrowth was significantly different among sainfoin accessions (*P<0.001*), but the year (*P=0.27*) and the year × accession interaction (*P=0.65*) did not affect regrowth of sainfoin (Table 4.1). The regrowth score ranged from 1.3 to 4.7 with a mean value of 3.6 (Figure 4.2). The five accessions with the highest regrowth scores were Remont (United States), Renumex (United States), SCO38401 (Canada), PI258773 (USSR) and Melrose (Canada).

4.5.2.4 Seed yield and 1000-seed weight

Seed yield and 1000-seed weight of sainfoin were significantly (*P<0.001*) different among the accessions. Compared to 2015, seed yield and 1000 seed weight were significantly (*P<0.001*) increased in 2016. The accession × year interaction was not significant for both seed yield (*P=0.46*) and 1000-seed weight (*P=0.71*) (Table 4.1). Seed yield varied greatly among the accessions from 5 g plant^{-1} (CN38745) to 64 g plant^{-1} (LRC3519), with an average of 29.7 g plant^{-1}. The five highest seed yielding accessions were PI440577 (Kazakhstan), SL895 (Canada), CN31800 (France), Nova (Canada) and SCO38401 (Canada) (Figure 4.2). The 1000-seed weight of sainfoin accessions varied from 13.3 (PI636518 from China) to 29.5 g (Svkpiestt from Slovakia) with an average of 22.6 g (Figure 4.2).

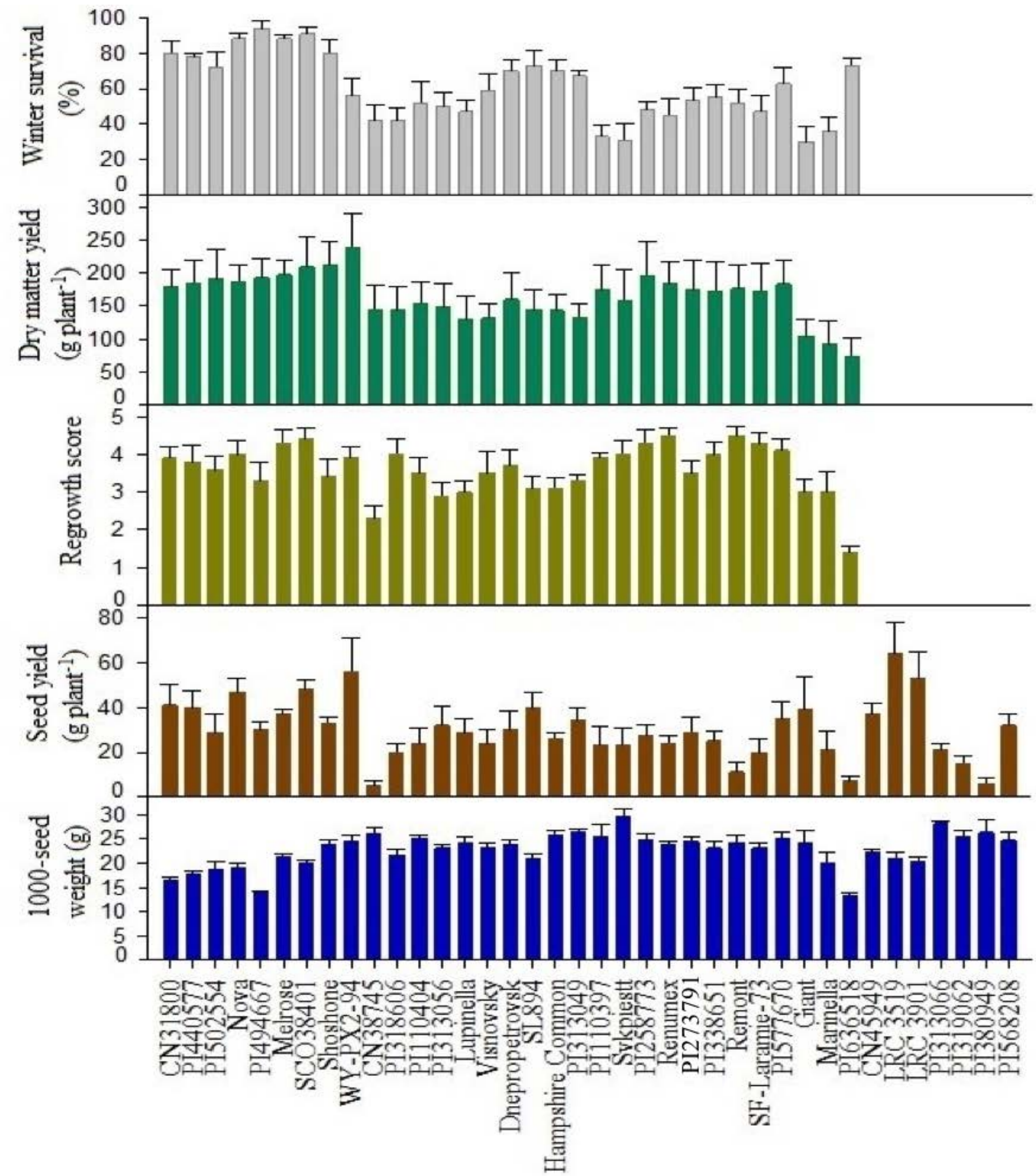

Figure 4.2 Two-year means (2015 and 2016) of winter survival (%), forage dry matter yield (g plant^{-1}), regrowth score, seed yield (g plant^{-1}) and 1000-seed weight (g) for sainfoin accessions grown at Saskatoon, SK, Canada (Bars are means ± Standard deviation).

4.5.2.5 Plant height and growth rate

The plant height and growth rate were highly significantly different among accessions ($P<0.001$) and between the two years (*P<0.001*), but the accession × year interaction was not significant for plant height (*P=0.78*) and growth rate (*P=0.11*) (Table 4.1). Plant height ranged from 39 cm (PI636518 from China) to 70 cm (Nova from Canada) with an average of 61 cm at the early flowering stage (Table 4.2). Even though plant growth habit was not directly measured, plants showed erect or semi-erect growth habit (spreading type). Growth rate of sainfoin was calculated from plant height recorded at two times at a 10-day interval. Growth rate ranged from 1.7 cm day^{-1} (PI636518 from China) to 2.7 cm day^{-1} (Nova and Melrose from Canada).

4.5.2.6 Spring vigor

There was highly significant (*P<0.001*) differences among the 38 sainfoin accessions for spring vigor, but it was not affected by accession × year interaction (*P=0.09*) (Table 4.1). A significant year (*P=0.005*) effect was due to more vigorous sainfoin growth in year 2016 than in 2015. Spring vigor score ranged from 2.0 (PI636518 from China) to 4.4 (WY-PX2-94 from United States) with an average of 3.5 (Table 4.2). The five accessions with high spring vigor score were WY-PX2-94 (United States), PI258773 (USSR), Melrose (Canada), Renumex (United States) and LRC3901 (Canada).

4.5.2.7 Days to flower

Days to flower was significantly different for accession (*P<0.001*), year (*P<0.001*) and their interaction (*P=0.002*) (Table 4.1). Days to flower ranged from 33 d (Hampshire Common from England) to 48 d (PI568208 from Turkey). The five late flowering accessions were PI568208

(Turkey), PI494667 (Romania), PI502554 (USSR), PI440577 (Kazakhstan) and CN31800 (France). The five early flowering accessions were Hampshire Common (England), Giant (England), PI313066 (Bulgaria), PI319062 (Spain) and Marinella (Slovakia) (Table 4.2).

4.5.2.8 Stem number

Stem number per plant was higher in 2016 than in 2015 ($P<0.001$). Stem number was significantly ($P<0.001$) different among accessions, but the year × accession interaction was non-significant ($P=0.21$) (Table 4.1). Stem number of individual accessions was in a range of 31 (PI380949) to 83 (PI440577) with an average value of 59 (Table 4.2). The five accessions with highest stem number were PI440577 (Kazakhstan), PI494667 (Romania), SCO38401 (Canada), WY-PX2-94 (United States) and PI502554 (USSR).

Table 4.2 Two year means for five agro-morphological characteristics measured on 38 sainfoin accessions in 2015 and 2016 at Saskatoon, SK, Canada

Accessions	Country	Continent	Plant Height (cm)	Growth rate (cm day^{-1})	Spring vigor	Days to Flower	Stem number
LRC 3519	Canada	North America	65	2.5	3.8	38	53
LRC 3901	Canada	North America	68	2.3	4.1	38	54
Melrose	Canada	North America	68	2.7	4.1	39	76
Nova	Canada	North America	70	2.7	3.9	38	63
Remont	United States	North America	66	2.2	3.7	37	50
Renumex	United States	North America	68	2.1	4.1	40	52
SCO38401	Canada	North America	65	2.6	4.0	40	80
SF-Laramie-73	United States	North America	61	2.2	3.7	37	55
Shoshone	United States	North America	65	2.5	3.9	37	63
SL895	Canada	North America	63	2.4	3.7	40	68
WY-PX2-94	United States	North America	61	2.5	4.4	38	79
CN31800	France	Europe	62	2.6	3.6	41	70
Giant	England	Europe	62	2.4	3.1	34	46
Hampshire Common	England	Europe	60	2.4	3.6	33	62
Lupinella	Italy	Europe	59	2.3	3.3	40	49
Marinella	Slovakia	Europe	53	2.0	2.4	35	46
PI273791	Ukraine	Europe	62	2.5	3.2	38	51
PI313049	Poland	Europe	63	2.3	3.7	36	51
PI313056	Norway	Europe	59	2.4	3.8	38	53
PI313066	Bulgaria	Europe	52	1.9	3.0	34	55
PI318606	Switzerland	Europe	56	2.1	3.5	37	58
PI319062	Spain	Europe	61	2.1	3.7	35	51
PI494667	Romania	Europe	54	2.4	3.9	43	82
Svkpiestt	Slovakia	Europe	60	2.2	3.7	40	58
Visnovsky	Czech republic	Europe	58	2.2	3.3	40	60
Dnepropetrovsk	USSR	Asia	56	2.1	3.5	37	65
CN38745	Turkey	Asia	54	2.0	3.8	41	63
CN45949	China	Asia	69	2.4	3.8	39	59
PI110397	USSR	Asia	67	2.3	4.0	37	47
PI110404	USSR	Asia	63	2.6	3.5	37	44
PI258773	USSR	Asia	67	2.6	4.1	39	57
PI380949	Iran	Asia	51	1.9	2.1	38	31
PI440577	Kazakhstan	Asia	65	2.6	4.0	42	83
PI502554	USSR	Asia	56	2.2	3.5	42	77
PI568208	Turkey	Asia	37	1.5	2.5	48	54
PI577670	China	Asia	66	2.5	3.6	40	63
PI636518	China	Asia	39	1.7	2.0	40	53
PI338651	Morocco	Africa	66	2.4	3.3	37	44
		Mean	60.7	2.3	3.6	38	59
		LSD[a] (0.05)	8.1	0.4	0.7	3.2	17.0
		SEM[b]	2.8	0.1	0.2	1.1	5.7
		CV[c]	12.1	11.8	15.2	7.2	20.1

[a] Least significant difference at $P<0.05$; [b] Standard error of means; [c] Coefficient of variation.

4.5.2.9 Nutritive value

Among sainfoin accessions, concentrations of CP (*P<0.001*), ADF (*P=0.002*) and NDF (*P<0.001*) varied significantly (Table 4.1). Compared to 2016, CP concentration was significantly (*P<0.001*) higher in 2015. There were significantly lower concentrations of ADF (*P<0.001*) and NDF (*P<0.001*) in 2015 than in 2016. The accession and year interaction was not significant for CP (*P=0.63*), ADF (*P=0.63*) and NDF (*P=0.76*). The concentration of CP for the accessions ranged from 134 (PI577670 from China) to 175 g kg^{-1} DM (PI636518 from China) with an average of 156 g kg^{-1} DM (Table 4.3). The concentration of NDF ranged from 376 (Svkpiestt from Slovakia) to 458 g kg^{-1} DM (PI577670 from China) with an average of 402 g kg^{-1} DM (Table 4.3). The concentration of ADF ranged from 349 (PI258773 from USSR) to 416 g kg^{-1} DM (PI577670 from China) with an average of 379 g kg^{-1} DM (Table 4.3).

Table 4.3 Two year means for concentrations (g kg^{-1} DM) of crude protein, neutral detergent fiber and acid detergent fiber measure on 31 sainfoin accessions in 2015 and 2016 at Saskatoon, SK, Canada

Accessions	Country	Continent	Crude Protein	Neutral Detergent Fiber	Acid detergent fiber
Melrose	Canada	North America	154	428	389
Nova	Canada	North America	148	425	401
Remont	United States	North America	151	400	368
Renumex	United States	North America	151	391	375
SCO38401	Canada	North America	169	383	379
SF-Laramie-73	United States	North America	154	397	367
Shoshone	United States	North America	162	423	384
SL895	Canada	North America	154	392	370
WY-PX2-94	United States	North America	151	415	383
CN31800	France	Europe	151	422	402
Giant	England	Europe	173	392	375
Hampshire	England	Europe	152	402	379
Lupinella	Italy	Europe	150	385	351
Marinella	Slovakia	Europe	171	378	358
PI273791	Ukraine	Europe	157	411	375
PI313049	Poland	Europe	159	401	385
PI313056	Norway	Europe	159	387	380
PI318606	Switzerland	Europe	164	390	381
PI494667	Romania	Europe	146	434	406
Svkpiestt	Slovakia	Europe	170	376	360
Visnovsky	Czech republic	Europe	137	391	364
CN38745	Turkey	Asia	160	406	381
Dnepropetrovsk	USSR	Asia	157	398	378
PI110397	USSR	Asia	146	416	388
PI110404	USSR	Asia	168	378	361
PI258773	USSR	Asia	160	385	349
PI440577	Kazakhstan	Asia	157	419	395
PI502554	USSR	Asia	165	429	394
PI577670	China	Asia	134	458	416
PI636518	China	Asia	175	356	349
PI338651	Morocco	Africa	139	405	376
		Mean	156	402	379
		LSD[a] (0.05)	18.9	36.3	30.1
		SEM[b]	7.5	12.9	10.8
		CV[c]	16.0	11.0	12.5

[a]Least significant difference at $P<0.05$; [b]Standard error of means; [c]Coefficient of variation.

4.5.3 Association among agro-morphological and nutritive traits

Correlations between traits are shown in Table 4.4. Forage DM yield had a positive correlation with plant height (r=0.82, $P<0.001$), stem number (r=0.75, $P<0.001$), growth rate (r=0.72, $P<0.001$), and spring vigor (r=0.43, $P<0.001$). Forage DM yield was negatively correlated with days to flower (r=−0.57, $P<0.001$), and CP (r=−0.62, $P<0.001$). As expected, forage DM yield was positively correlated with NDF (r=0.71, $P<0.001$) and ADF (r=0.74, $P<0.001$). A non-significant (r=−0.05, $P=0.53$) correlation was found between forage DM yield and seed yield. On the other hand, seed yield had significant positive correlation with spring vigor (r=0.32, $P<0.001$). Seed yield was negatively correlated with 1000-seed weight (r=−0.16, $P=0.01$), indicating a high seed yield for sainfoin accessions with small seed size. Regrowth showed positive correlations with spring vigor (r=0.42, $P<0.001$) and forage DM yield (r=0.30, $P<0.001$).

Forage nutritive value revealed significant correlation with some observed traits. Crude protein showed significant positive correlation with days to flower (r=0.50, $P<0.001$), but negatively correlated with plant height (r=−0.71, $P<0.001$), spring vigor (r=−0.29, $P<0.001$), stem number (r=−0.59, $P<0.001$), growth rate (r=−0.64, $P<0.001$), NDF (r=−0.74, $P<0.001$), and ADF (r=−0.72, $P<0.001$). Furthermore, ADF had significant correlations with plant height (r=0.73, $P<0.001$), spring vigor (r=0.18, $P=0.02$), days to flower (r=−0.54, $P<0.001$), stem number (r=0.74, $P<0.001$), growth rate (r=0.72, $P<0.001$). Similarly, NDF had significant correlations with plant height (r=0.68, $P<0.001$), spring vigor (r=0.31, $P<0.001$), days to flower (r=−0.45, $P<0.001$), stem number (r=0.61, $P<0.001$), growth rate (r=0.65, $P<0.001$).

Table 4.4 Coefficient of correlation (r) between 13 traits observed on 38 sainfoin accessions grown in 2015 and 2016 at Saskatoon, SK, Canada

	PH	SV	DF	SN	WS	SY	TSW	GR	CP	NDF	ADF	DMY	RG
Plant height	1[a]	***	***	***	ns	ns	***	***	***	***	***	***	*
Spring vigor	0.47	1	***	***	ns	***	**	***	***	***	*	***	***
Days to flower	−0.75	−0.30	1	***	*	ns	***	***	***	***	***	***	ns
Stem number	0.73	0.38	−0.53	1	*	ns	ns	***	***	***	***	***	ns
Winter survival	−0.04	0.11	0.17	0.17	1	ns	***	ns	ns	ns	ns	ns	ns
Seed yield	−0.03	0.32	0.08	−0.04	0.09	1	*	ns	ns	ns	*	ns	ns
1000-seed weight	0.39	0.19	−0.40	0.05	−0.37	−0.16	1	***	***	*	ns	***	ns
Growth rate	0.88	0.30	−0.60	0.71	0.03	−0.01	0.28	1	***	***	***	***	ns
CP	−0.71	−0.29	0.50	−0.59	−0.11	0.11	−0.25	−0.63	1	***	***	***	ns
NDF	0.68	0.31	−0.45	0.61	0.14	−0.06	0.16	0.65	−0.76	1	***	***	**
ADF	0.73	0.18	−0.54	0.74	0.13	−0.16	0.13	0.72	−0.73	0.87	1	***	ns
DM yield	0.82	0.43	−0.57	0.75	0.09	−0.05	0.29	0.72	−0.62	0.71	0.74	1	***
Regrowth	0.18	0.42	0.00	0.02	0.13	0.12	0.14	0.06	−0.12	0.21	0.10	0.30	1

PH=plant height; SV=spring vigor; DF=days to flower; SN=stem number; WS=winter survival; SY=seed yield; TSW=1000-seed weight; GR=growth rate; CP=crude protein; NDF=neutral detergent fiber; ADF=acid detergent fiber; DMY=dry matter yield; RG=regrowth.

[a] Coefficient of correlation (r); ***, significant at $P<0.001$; **, significant at $P<0.01$; *, significant at $P<0.05$; ns, not significant.

4.5.4 Clusters of sainfoin accessions based on agro-morphological and nutritive traits

There were four principal components with eigenvalues great than 1 which accounted for 41%, 23%, 10%, and 8% of the total variation, respectively (Table 4.5). The first two components of the PCA explained 64% of the total observed variation. The relative magnitude of eigenvectors in the first component (PC1) indicated that winter survival, growth rate, spring vigor, stem number, seed yield, DM yield, regrowth, NDF and ADF were the most important traits for classifying populations into clusters. The second component (PC2) was strongly correlated with plant height, 1000-seed weight. Crude protein was an important variable in the third component (PC3) and days to flower was an important variable in the fourth component (PC4) (Table 4.5).

Table 4.5 Eigenvectors from the first four principal components for 13 traits used to classify 31 sainfoin accessions

Variables	PC1[a]	PC2	PC3	PC4
Winter survival	**0.61**	−0.60	0.17	0.19
Plant height	0.66	**0.67**	−0.03	0.12
Growth rate	**0.76**	0.29	0.24	0.35
Spring vigor	**0.78**	0.38	0.11	−0.28
Days to flower	0.36	−0.53	0.06	−**0.64**
Stem number	**0.69**	−0.52	0.28	−0.21
Seed yield	**0.71**	0.04	0.38	0.40
1000-seed weight	−0.14	**0.84**	−0.14	−0.09
Dry matter yield	**0.87**	0.19	0.08	−0.26
Regrowth	**0.63**	0.54	0.03	−0.23
Crude protein	−0.50	−0.12	**0.69**	0.07
Neutral detergent fiber	**0.73**	−0.27	−0.52	0.14
Acid detergent fiber	**0.57**	−0.55	−0.39	0.25
Eigenvalue	5.36	3.00	1.25	1.07
Proportion (%)	41.22	23.08	9.62	8.26
Cumulative (%)	41.22	64.29	73.91	82.17

[a] The bold and underlined coefficients have significant correlation with the relevant component in the PCA.

The UPGMA cluster analysis using 13 agro-morphological and nutritive value traits based on Euclidean distance grouped the 31 sainfoin accessions into three main clusters (Figure 4.3).

The mean comparison among clusters is shown in Table 4.6. The first cluster included nine accessions which were characterized by high winter survival, high growth rate, good spring vigor, high stem number, good regrowth, high DM yield and higher seed yield. The second cluster comprised 19 accessions characterized by high 1000-seed weight. Three accessions aligned together in the third cluster and depicted poorer performance in terms of forage DM yield, but contained higher CP concentration.

Table 4.6 Mean comparison of agro-morphological and nutritive traits in cluster analysis of 31 sainfoin (*Onobrychis viciifolia* Scop.) accession

Traits	F probability	Mean[a]		
		Cluster I (n=9)	Cluster II (n=19)	Cluster III (n=3)
Winter survival (%)	***	80.6[a]	52.6[b]	46.4[b]
Plant height (cm)	**	62.9[a]	61.8[a]	51.3[b]
Growth rate (cm day^{-1})	***	2.5[a]	2.3[b]	2.1[b]
Spring vigor	***	3.9[a]	3.6[a]	2.5[b]
Days to flower	*	40.1[a]	38.1[ab]	36.4[b]
Stem number	***	74.8[a]	55.2[b]	48.4[b]
Seed yield (g plant^{-1})	***	40.0[a]	25.2[b]	22.3[b]
1000-seed weight (g)	***	19.6[b]	24.5[a]	19.2[b]
Dry matter yield (g plant^{-1})	***	199.1[a]	159.1[b]	90.0[c]
Regrowth scores	**	3.8[a]	3.7[a]	2.5[b]
Crude protein (g kg^{-1} DM)	**	155.9[b]	153.8[b]	172.7[a]
Neutral detergent fiber (g kg^{-1} DM)	**	419.6[a]	398.3[b]	383.0[b]
Acid detergent fiber (g kg^{-1} DM)	**	392.4[a]	373.8[b]	373.6[ab]

[a] Means with same letters within the row for each trait are not significantly different (*$P>0.05$*).

The dispersion of 31 accessions based on first and second components are expressed in Figure 4.4. The first axis (X) was formed by DM yield, seed yield, growth rate, spring vigor (positive coefficient) and CP (negative coefficient). The accessions within the first cluster lie on the right of the X axis and have highest values for DM yield. The second axis (Y) was comprised of 1000-seed weight and plant height (positive coefficient) and days to flower, ADF, NDF and stem number (negative coefficient). The accession from the third cluster lies at the bottom of the Y axis due to being the shortest accession with the lowest 1000-seed weight (Figure 4.4). The PCA based on these 13 traits did not clearly differentiate accessions, although variation among accessions were highly significant. The PCA reasonably coincided with the groupings of the UPGMA dendrogram with PI636518 (China) being the most unique accession.

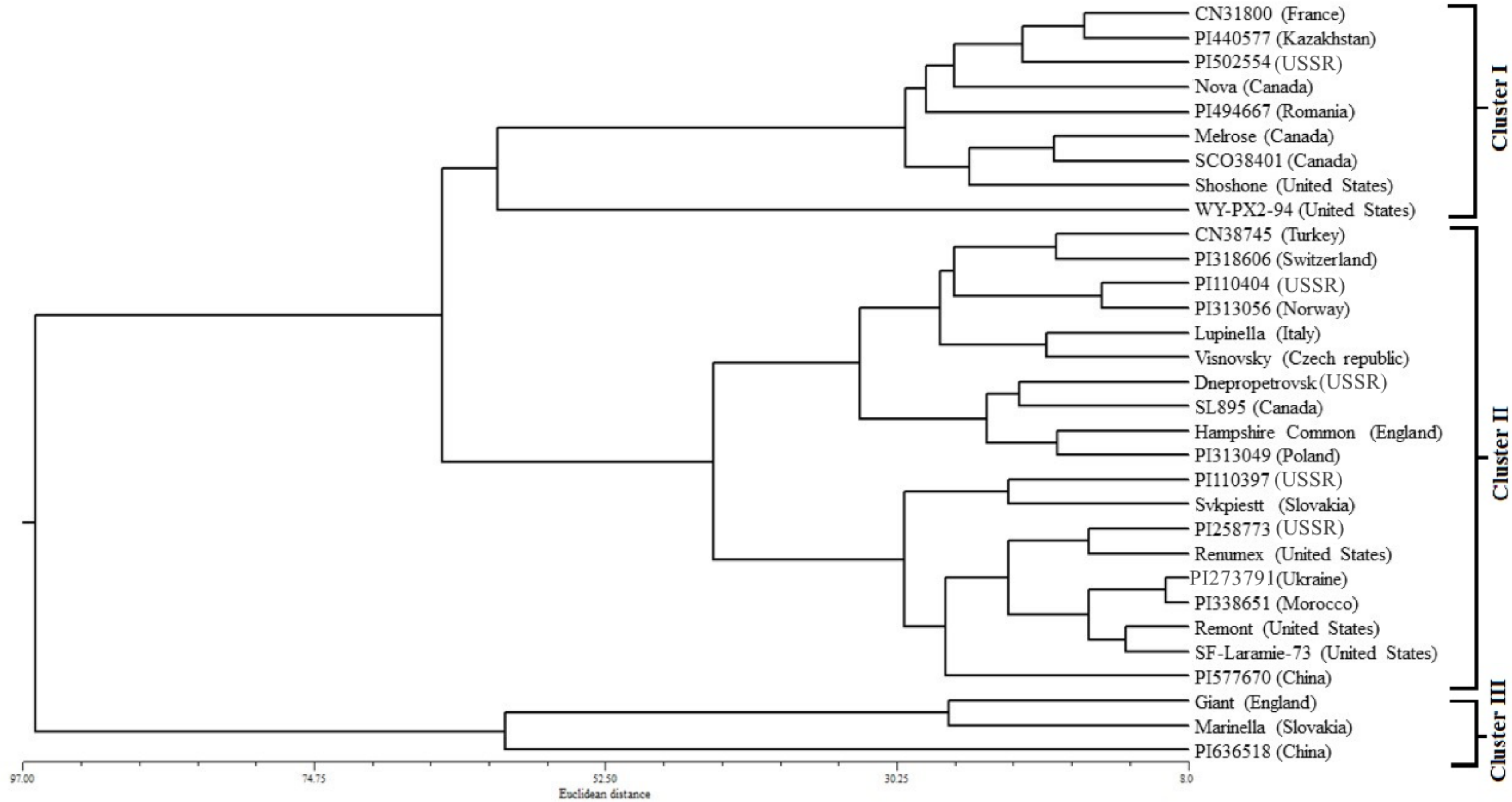

Figure 4.3 Dendrogram of the 31 sainfoin accessions revealed by UPGMA cluster analysis based on 13 agro-morphological and nutritive values (2-yr means).

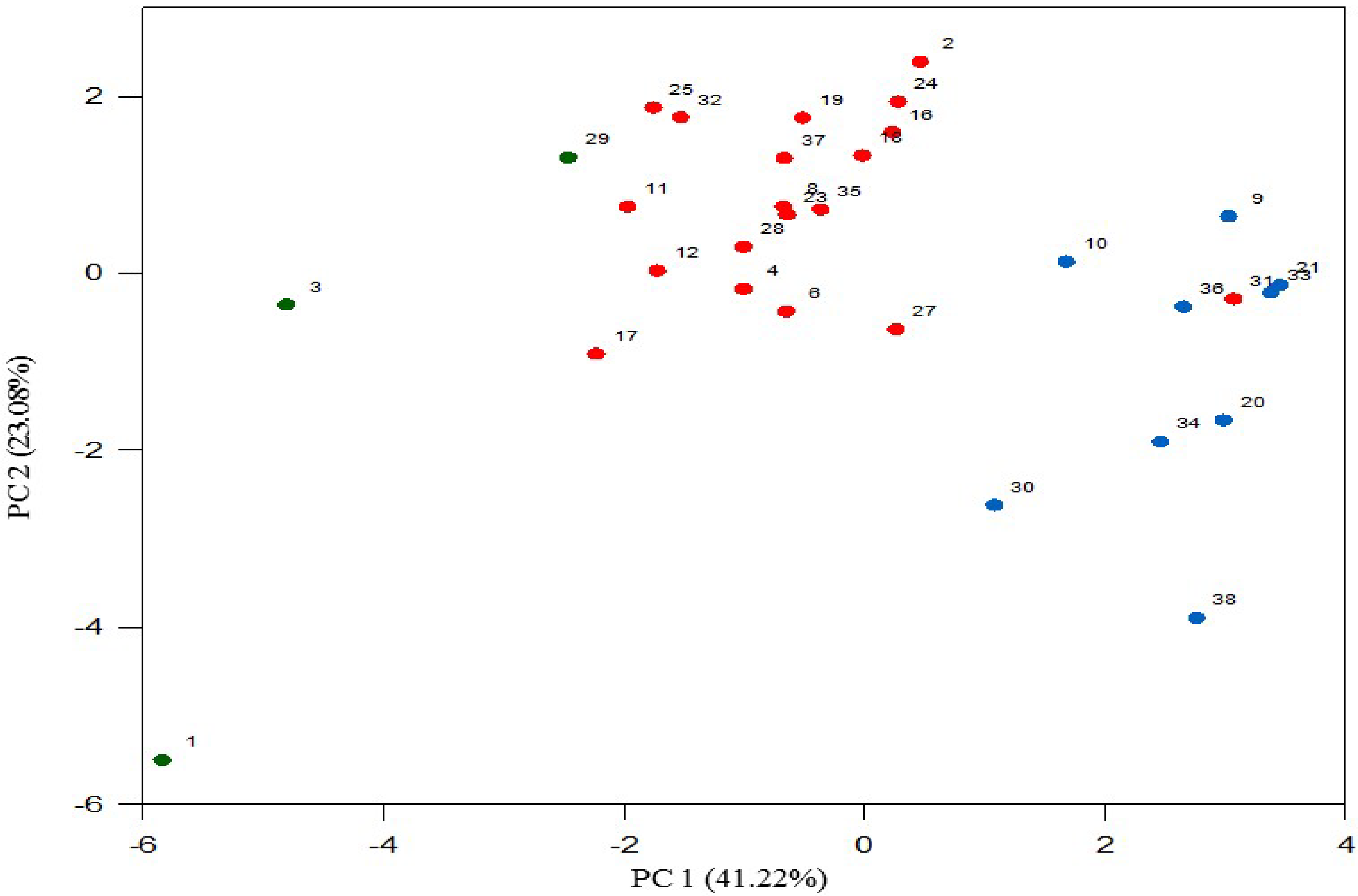

Figure 4.4 Cluster plot created based on principal component analysis of 31 sainfoin accessions using 13 traits. Accessions from the first cluster (blue), second cluster (red) and third cluster (green) are highlighted with different colors. Each individual plant is labeled with a code representing its accession (Table 3.1).

4.6 Discussion

In this study, all sainfoin plants in the third year (2016) produced higher forage DM yield and were taller, more dense and vigorous than in 2015. It was not surprising, because perennial forage crops undergo development over years resulting in increases in size and height (Jafari et al. 2003). Similar phenomenon in sainfoin has been reported (Martiniello 1998; Mohajer et al. 2013). In this study, this trend across the two growing seasons was evident for almost all agronomic traits, which may in part be due to higher rainfall during the 2016 growing season. In this study, the year × accession interaction was not significant for any traits except days to flower, indicating performance ranking of all accessions did not change over years. This is important for identifying germplasm with superior performance under different growing environments. In this study, WY-PX2-94 (United States), Shoshone (United States), SCO38401 (Canada) and Melrose (Canada) were the best lines for forage production over the two years. The significant accession × year for days to flower showed that flowering of sainfoin is influenced by environmental factors. Delgado et al. (2008) reported that water stress inhibits flowering to certain extent, which may be the reason for significant year × accession effect on days-to-flower as the growing season in 2015 was much drier than in 2016. Sainfoin accessions contained higher concentrations of CP, lower concentrations of ADF and NDF in 2015 than in 2016. This may be in part due to an increase in total DM yield in 2016, which diluted the concentrations of nutritive values (Lemaire et al. 1994). Parker and Moss (1981) reported Holstein, average daily gain of 0.96 kg for Holstein heifer feeding on 7.4 kg of sainfoin hay containing 150 g kg^{-1} DM protein, 436 g kg^{-1} DM NDF and 393 g kg^{-1} DM ADF. The majority of sainfoin accessions tested in our study would provide similar nutritive values (156 g kg^{-1} DM CP, 402 g kg^{-1} DM NDF and 379 g kg^{-1} DM ADF) to Parker and Moss (1981) study.

Increasing forage DM yield is an important goal in sainfoin breeding program. This study showed significant positive correlations of DM yield with plant height and stem number, which is in agreement with previous studies on sainfoin (Turk and Celik 2006; Nakhjavan et al. 2011; Mohajer et al. 2012; Jafari et al. 2014). This also indicates that selection for high DM yield of sainfoin can be accomplished by selecting tall and dense plants. In addition, days to flower had a negative correlation with stem number and DM yield in sainfoin, which was similar to findings in alfalfa, perennial ryegrass (*Lolium perenne* L.), tall fescue (*Festuca arundinacea* Screb.) (Martiniello 1998) and cocksfoot (*Dactylis glomerata* L.) (Martiniello 1998; Jafari and Naseri 2007). This suggested that selection of early flowering types in sainfoin could lead to an increase in stem number and DM yield. However, DM yield had negative correlation with CP and positive correlation with NDF and ADF, which is common in many forage species (Martiniello 1998; Turk and Celik 2006; Mohajer et al. 2013). This may present a practical challenge to simultaneously improve forage DM yield and nutritive value in sainfoin breeding.

The 31 sainfoin accessions were clearly grouped into three main clusters based on their agro-morphological traits and nutritive value. The accessions from East Asia and Europe were distributed in all three clusters showing a wide range in agronomic performance. The accessions having above average performance in terms of winter survival, DM yield, seed yield and stem number grouped in one cluster. This cluster was comprised of accessions from North America. However, a study by Hayot-Carbonero (2011) in United Kingdom found that Eastern European accessions had the highest forage DM yield. Similarly, Iranian accessions out yielded all collected germplasm in a study conducted in the Middle Eastern region (Zarrabian et al. 2013). Giant (England), Hampshire common (England), Marinella (Italy), and Dnepropetrovsk (USSR), all of which are European sainfoin landraces, may perform well in their respective environments, but they showed relatively poor agronomic performance in Western Canada. This suggested that selection for regional adaptation in sainfoin is an important consideration in future breeding efforts. The accessions in the second cluster have higher than average 1000-seed weight. The accessions in the third cluster have characteristics of poor winter survival, shortness, few stems, but have high nutritive value (high CP and low NDF and ADF).

Previous studies have also revealed wide variations in sainfoin germplasm for most of the agro-morphological traits studied here. Delgado et al. (2008) reported high variation in 44 Spanish sainfoin accessions that formed two clear clusters according to flower number in the year of seeding and regrowth rate. Zarrabian et al. (2013) grouped 56 sainfoin accessions (46 Iranian accessions and 10 worldwide collections) into three clusters using 13 agro-morphological traits. Mohajer et al. (2013) reported three clusters of 12 sainfoin accessions from Iran using 17 agro-morphological and nutritional traits. Nakhjavan et al. (2011) also observed wide genetic variation in 34 sainfoin populations from Iran and grouped them in four clusters based on forage DM yield and nutritional traits. The results of these studies were in agreement with the current study of 38 accessions. Development of synthetic varieties is the most effective breeding method in outcrossing perennial forages (Vogel and Pedersen 1993). The high variation in sainfoin accessions is valuable for developing varieties with high DM yield, high seed yield and good nutritive value. Future multiple-location studies using the selected half-sib families of sainfoin accessions from this study, especially at locations varying in precipitation, would be useful to further explain the contribution of genotype and environment to total phenotypic variation.

Chapter 5. Effects of seed size, seed pod removal and temperature on seed germination of sainfoin (*Onobrychis viciifolia* Scop.)

5.1 Abstract

Sainfoin (*Onobrychis viciifolia* Scop.) is a perennial leguminous forage crop used for hay production or grazing. Sainfoin has a single seed per pod and it is generally seeded without removing the seed pod. The objectives of this study were to evaluate the effects of seed size and pod removal on sainfoin seed germination under different temperatures. The experimental design was a 3 (seed sizes class) × 3 (temperature) × 2 (pod removal) factorial arrangements in a randomized complete block design with four replications. Seed size ($P<0.001$), seed pod removal ($P<0.001$), temperature ($P=0.047$) and interaction between seed size × seed pod removal ($P<0.001$) had significant effects on sainfoin seed germination. Cumulative germination was the highest at day/night temperatures of 20/10°C and 15/5°C. Germination was increased after seed pod removal. Final seed germination was 2.4%, 91.6% and 81.0%, respectively, for three accessions with small, medium and large seed size classes. An additional experiment was designed to examine variation in seed germination among seed size classes within a cultivar. However, final seed germination of small and large seed size classes for Melrose sainfoin was similar (76.5% and 80%, respectively).

5.2 Introduction

Sainfoin (*Onobrychis viciifolia* Scop.) is adapted to a wide range of climatic and soil conditions. It is considered an important forage legume from the hot and dry Mediterranean region to northern latitudes with severe winters such as Western Canada (Frame et al. 1998). Sainfoin is an outcrossing species mostly pollinated by insects (Goplen et al. 1991). Each axillary stem has an inflorescence comprised of around 80 pinkish red flowers. Each flower can produce a kidney-shaped seed, varying from yellowish green to dark brown or olive in color (Goplen et al. 1991). Unlike alfalfa (*Medicago sativa* L.), sainfoin seeds are borne singly in pods. Seed can be planted with pods removed (milled) or intact (unmilled), but in most cases pods are left intact (Thomson 1951). Hard seed pods can increase mechanical restriction of embryo growth and interfere with water uptake and gas exchange which results in slow germination (Carleton et al. 1968; Mayer and Shain 1974; Ditterline and Cooper 1975). In addition, seedlings emerging from seeds with pod intact had a high probability of fungal (*Alternaria* and *Fusarium* spp.) infestation (Ditterline and Cooper 1975). Also, slow and non-uniform seedling growth and high weed infestations may be other limiting factors for sowing seed with pod (Noorbakshian et al. 2011a). Removal of seed pods is possible, but it may cause seed injury during the process and increase seed processing cost. There are limited but conflicting views about germination of sainfoin seeds with respect to seed pod removal. Wiesner et al. (1968) and Noorbakshian et al. (2011a) reported pod removal enhanced sainfoin seed germination, but Chen (1992) found no significant improvement of germination.

Beside seed pod removal, Singh et al. (2009) reported seed size is an important parameter in seedling vigor and stand establishment of forage legumes. Seed size represents the amount of food reserves available during germination. Seedling vigor has been positively correlated with seed size in sainfoin and some other legumes (Carleton and Cooper 1972; Fransen and Cooper 1976; Singh et al. 2009). However, a major limitation to the wider use of sainfoin is its large seed size which results in higher costs for stand establishment than for other forage legumes. Sainfoin seed size is about six to seven times larger than many other common forage legumes, such as alfalfa or clover species (Thomson 1951). In addition, limited information is available on the optimum temperature range for seed germination of sainfoin. Smoliak et al. (1972) reported a wide optimum temperature range for sainfoin seed germination. Carleton et al. (1968) found that 15–20°C was the optimum temperature range for sainfoin seed germination. Seed germination rate usually increases linearly with increases in temperature up to an optimum point, but then declines (Steinmaus et al. 2000; Bradford 2002). The objective of this study was to determine the effects of seed size, seed pod removal and temperatures on sainfoin seed germination.

5.3 Materials and methods

Sainfoin seeds harvested from the field nursery grown in the 2015 growing season were used for this study. For Experiment I, three sainfoin accessions with different seed sizes based on their 1000-seed weight were selected for the germination test. The three accessions were Svkpiestt (large, 27.8g), Shoshone (medium, 22.3g) and PI636518 (small, 12.1g). Seed viability tests were conducted for each seed size class using the tetrazolium chloride test (Grabe 1970). The results of the tetrazolium chloride test showed that the large and medium size seed classes had 100% viable seeds, whereas small size seed class had 77% viable seeds. The experimental design was $3 \times 3 \times 2$ factorial arrangements in a randomized complete block design with four replications. Experimental factors were temperature (15/5°C, 20/10°C and 25/15°C), seed size class (large, medium and small) and seed pod removal (with or without seed pod). Germination tests were carried out in germination cabinets under day/night (12/12 h) temperatures of 15°C/5 °C, 20°C/10°C and 25°C/15°C. Fifty seeds were imbibed on top of two layers of filter paper (Whatman 597) in 9 cm sterilized plastic petri dishes moistened by 5 ml of distilled water. The petri dishes were enclosed and sealed in polyethylene bags to prevent desiccation. Germination counts was made daily for 14 days. Seeds with a radical greater than 2 mm were considered germinated and removed daily. The experiment was repeated twice.

From Experiment I significant differences in germination performance were found among three seed-size classes representing different accessions. An additional experiment was designed to examine variation in seed germination among seed size classes within a cultivar. Seed pod was removed prior to the study. Seeds from the sainfoin cultivar, Melrose, were separated into large (16.2g) and small (10.8g) seed size classes based on 1000-seed weight. The germination experiment was conducted at the optimum day/night (12/12 h) temperature of 20/10°C with four replications following similar procedures as described in Experiment I.

5.4 Statistical Analysis

Data were analyzed using the R software package, version 3.3.2 (R core team 2016). Data were subjected to analysis of variance (ANOVA) using the Mixed model procedure with temperature, seed size, seed pod removal and their interactions as fixed effects, and replication and experimental run as random effects. Means comparison was conducted using the least significant difference (LSD) post-hoc test at $P<0.05$ level.

5.5 Results

Analysis of variance showed that temperature ($P=0.047$), seed size class ($P<0.001$) and seed pod removal ($P<0.001$) had significant effects on final seed germination of sainfoin. In addition, interaction of seed size class × seed pod removal ($P<0.001$) had a significant effect on final seed germination, but temperature × seed pod removal and temperature × seed size class, temperature × seed size class × seed pod removal did not significant effects on sainfoin seed germination.

Final seed germination was higher at 20/10°C than at 25/15 °C (Table 5.1), but no difference was found between 15/5°C and 20/10°C, or between 15/5°C and 25/15°C (Table 5.1).

Table 5.1 Final seed germination (%) of sainfoin as affected by temperature, seed size class and seed pod removal

Treatment	Levels	Germination[a] (%)
Temperature	15/5°C	58.0[ab]
	20/10°C	60.0[a]
	25/15°C	56.8[b]
	SEM[b]	3.35
	P value	0.047
Seed size class	Large	81.0[b]
	Medium	91.6[a]
	Small	2.4[c]
	SEM	3.35
	P value	<0.001
Seed pod	without pod	62.8[a]
	with pod	53.9[b]
	SEM	3.31
	P value	<0.001

[a]Means with the same lower case letters within the column for each treatment are not significantly different ($P>0.05$);
[b]Standard error of means.

Final seed germination was 2.4%, 91.6% and 81% for small (PI636518), medium (Shoshone), and large (Svkpiestt) seed size classes, respectively. Seed germination started on the first day for medium and large seed size classes, but the majority of seeds in the small seed size class were not germinated, indicating a high degree of physical dormancy (Data not shown). Seed germination was significantly ($P<0.001$) increased after seed pod removal (62.8%) as compared to the intact seeds (53.9%).

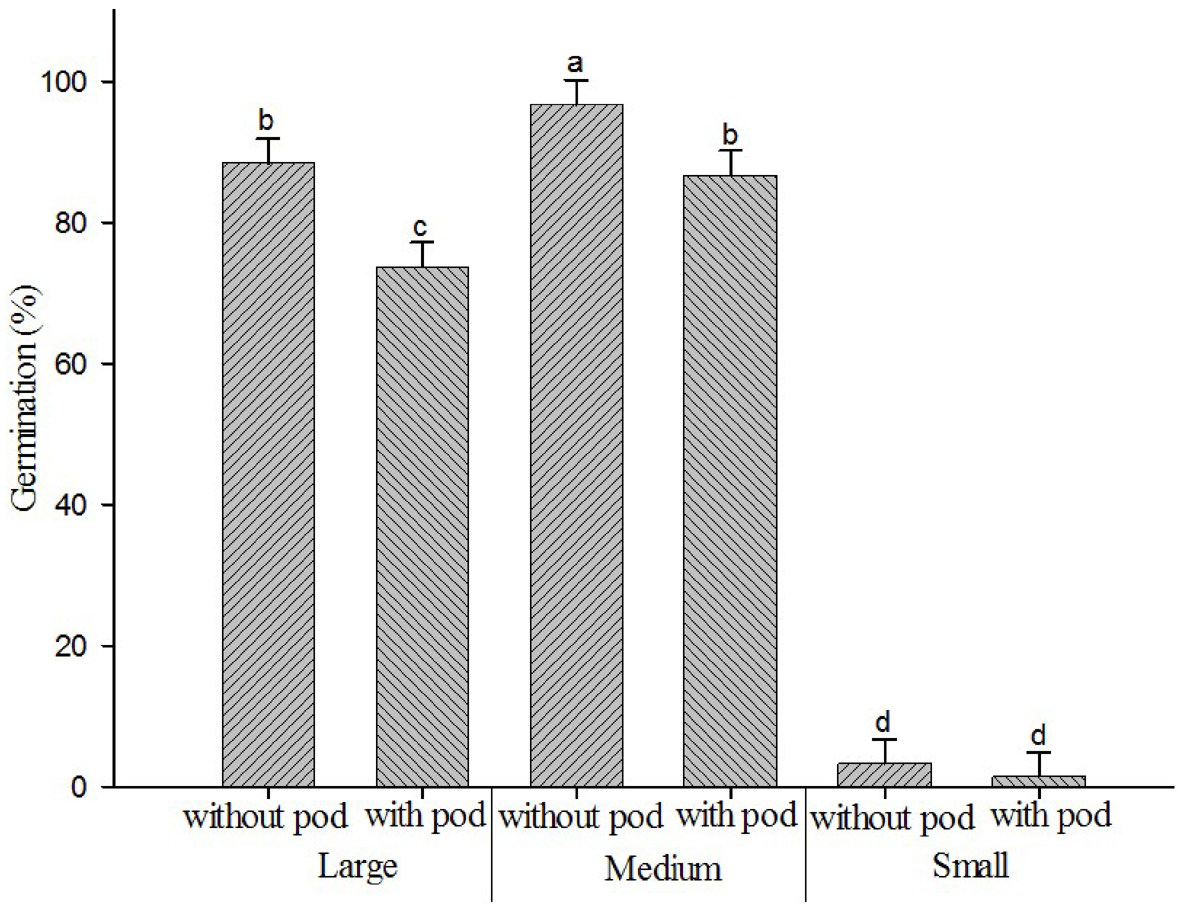

Figure 5.1 Interaction of seed pod removal and seed size class on final germination (%) of sainfoin seed. Bars with different lower case letters (a–d) are significantly different (P<0.05).

Seed size class × seed pod removal interaction was mainly due to the non-significant effect of seed pod removal in small seed size class (Figure 5.1). Seed pod removal, however, significantly increased final germination in large and medium size seed classes (Figure 5.1).

Seed size classes selected from the same cultivar, Melrose, did not affect seed germination (Table 5.2).

Table 5. 2 Final seed germination (%) of two different seed size classes of sainfoin cultivar Melrose

Treatment	Germination[a] (%)
Large	76.5[a]
Small	80.0[a]
SEM[b]	1.33
P value	0.07

[a]Means with the same lower case letters within the column for each treatment are not significantly different (*P<0.05*);
[b]Standard error of means.

5.6 Discussion

This study showed that high seed germination rate of sainfoin occurred at 20/10°C and 15/5°C, which is similar to the previous report by Carleton et al. (1968). This study also supported the finding of Smoliak et al. (1972) who reported that sainfoin had a wide range of optimum temperatures for seed germination. Germination of the three sainfoin accessions with different seed sizes was significant different, which may be due in part to genetic differences. Hayot-Carbonero (2011) found seed germination of different sainfoin germplasm varied from 0–90%, with higher germination of large seed size classes reported (Noorbakshian et al. 2011b). This was not fully supported by the current study as the medium seed size class had higher germination than the small and large seed sized classes. Black (1959) reported that the proportion of hard seed increases as seed size decreases, which could be a reason for low germination of the small size seed class. Even though the small size seed class had low germination rate, the seed viability rate was 77% based on the TZ test. It is interesting to note that seed size did not affect seed germination of sainfoin if they were selected from the same cultivar. While environment and position of the seed in the inflorescence affect seed size in plant species (Galloway 2001; Vallius 2000), seed size has generally been found to be a moderately heritable trait. The size of the seeds may be the result of either genetic or environmental factors or combination of both (Carleton and Cooper 1972; Cash and Ditterline 1996).

The result was in agreement with previous studies suggesting that pod removal can enhance seed germination of sainfoin (Wiesner et al. 1968; Noorbakshian et al. 2011a). Thomson (1951) suggested that low germination of seeds with pods was due to failure of the radicle to penetrate the pods. Carleton et al. (1968) found that seed pods slow seed water absorption by 4–5 h. A 1–2 d delay in germination was also observed in this study from seeds with the pod intact. However, Chen (1992) found no significant difference in germination between sainfoin seeds with or without pods under field conditions.

In conclusion, seed pod removal increased germination of sainfoin seed in this study. Non-significant differences in germination rate were observed for two seed size classes selected from Melrose, suggesting that it may be possible to decrease seed size without reducing germination.

Chapter 6. General discussion and conclusions

This M.Sc. research project was designed to provide genetic diversity and relationship information for 38 sainfoin accessions representing 20 different countries from four different continents. This research also evaluated agronomic and phenotypic characteristics, such as plant height, growth rate, spring vigor, days to flower, stem number, seed yield, 1000-seed weight, DM yield, regrowth, winter survival and nutritive value (CP, ADF and NDF) in a field nursery. In addition, the effects of seed size, seed pod removal on seed germination under different temperatures were evaluated.

The AFLP study showed wide variations among sainfoin accessions, but cluster analysis showed no clear clustering of accessions based on geographic locations. Accessions were somewhat grouped into sub-clusters on the basis of their respective pedigree. Therefore, the first hypothesis "sainfoin accessions are genetically diverse, but the accessions from similar geographical region are genetically related" was partially accepted (see Chapter 3). Sainfoin accessions showed large variations for all measured agro-morphological traits and nutritive values in the study. In addition, the majority of accessions from North America had superior agronomic performance than the accessions from Asia or Europe, indicating a high regional adaptation for agro-morphological traits. The second hypothesis "agro-morphological characteristics and nutritive values differ among sainfoin accessions, but accessions adapted to the North America have superior agronomic performance" was accepted (See Chapter 4). Third hypothesis "seed pod removal and seed size increase will increase germination rate under different temperatures" was partially accepted according to the results from germination experiment (see Chapter 5). Final seed germination varied across different temperature regimes and seed pod removal enhanced seed germination. However, seed germination was not found to be directly proportional to seed size, because the medium sized seed class showed greater germination than large or small seed size class.

Agro-morphological characteristics differed among sainfoin accessions in this study, which was in agreement with previous studies (Delgado et al. 2008; Nakhjavan et al. 2011; Mohajer et al. 2013; Zarrabian et al. 2013; Jafari et al. 2014). In this study, wide variation of winter survival was observed among the accessions. In northern temperate regions, in particular in Western Canada, winter survival is one of the key characters to consider for new germplasm introductions. The sainfoin accessions in this study are from different climatic regions and a relatively large number of plants (43%) did not survive their first winter. This was evident for 7 of 38 accessions and may have resulted in uneven competition among the individuals for soil nutrients and moisture. However, the shattered seeds from the 2015 field season replaced the dead plants in 2016 which decreased the uneven distribution of plants. Another major yield component of forage crops is plant height. This study found average plant height of the sainfoin accessions was 70 cm, but a few individuals reached as tall as 93 cm, similar to previous reports by Frame et al. (1998) and Hayot-Carbonero (2011). The average height of Canadian sainfoin cultivars was 83 cm for Nova and 91 cm AAC Mountainview (Acharya 2015). In addition, it also noticed that some accessions has prostrate growth habit, such as PI636518 (China), PI494667 (Romania) and PI568208 (Turkey). The early flowering accessions produced greater forage DM yield than the later flowering accessions. The early accessions started to flower in the first week of June while the late flowering accessions initiated flowering in the last week of June. Even though there was significant year × accession interaction for days to flower, certain accessions showed consistent early or late flowering. For example, accession PI568208 (Turkey) was a late flowering accession in both years which flowered in the last week of June in 2015 and in the second week of June in 2016. Stem number per plant was highly variable among the sainfoin accessions, which was positively correlated to forage DM yield.

Even though improvement of forage DM yield is one of the most important breeding goals in forage breeding, high seed yield and reduced seed size are also considered important for sainfoin genetic improvement. A large seed size has been a major factor for high seed cost for sainfoin, which discouraged wider adoption of this species by beef and forage producers. In this study, seed yield and 1000-seed weight varied greatly among the sainfoin accessions, which will be important to further select small seed size. The seed size of accession PI636518 (China) was found about 1/3 of the Canadian cultivars. The accession PI636518 was unique, as illustrated by two separate PCA analysis using agro-morphological or AFLP markers. Even though the ploidy level of this accession was not determined, it was speculated that the small seed size may be due to a difference in ploidy (Oakwood et al. 1993; Bretagnolle et al. 1995). Non-significant germination performance among different seed size within an accession indicated the possibility for selecting small seed size without affecting seed germination. However, the relationship of seed size with seedling vigor and spring vigor still remained unanswered in this study.

Sainfoin-alfalfa mixtures are a useful method to reduce pasture bloat in grazing animals, but regrowth of old sainfoin cultivars is much slower than alfalfa (Acharya et al. 2013). Regrowth is an important trait for developing a persistent sainfoin cultivar under such a sainfoin-alfalfa mixture. The majority of plant introductions regrow slower than the Canadian adapted cultivars, Nova or Melrose. However, it may be possible make improvements for regrowth of these cultivars, as they showed high genetic and phenotypic variation.

In this study, genetic relationships among the sainfoin accessions as revealed by agro-morphological traits and nutritive values are partially consistent with the pattern detected by the

AFLP markers. This supported the idea that AFLP markers are suitable for genetic diversity evaluation of sainfoin. For example, accessions from the United States, WY-PX2-94 and Shoshone, were clustered closely together, and two other cultivars from the United States, Remont and Renumex, were also clustered together in both analyses, supporting their grouping based on pedigree and origin. However, the two methods were not always consistent. For example, Canadian cultivar Melrose was developed from USSR germplasm, and clustered with USSR accessions (PI110397 and PI110404) in the AFLP analysis, but it was clustered with another USSR accession PI502554 in the phenotypic study.

The selection of parental combinations is important for developing synthetic cultivars. Accessions identified as having high genetic variation and superior agro-morphological traits could be used for developing new synthetic varieties. The assayed sainfoin accessions have high within-accession genetic variation in addition to high agro-morphological traits, suggesting that sampling a small number of genotypes would capture a large genetic variation. In this study, several promising accessions were identified which could be used to develop synthetic cultivars with high forage yield, improved persistence and nutritive value. The top four highest DM yielding accessions, WY-PX2-94, Shoshone, SCO38401, and Melrose, are from North America. On the contrary, the five of nine accessions with high agronomic performance (CN31800, PI440577, PI502554, Nova and SCO38401) from cluster I (Figure 4.3), were also grouped together in the AFLP analysis (Figure 3.1). This suggests there may be certain common genes governing the superior agronomic traits, which could be further exploited by genotyping plants from the accessions.

In North America, few forage breeders are working on sainfoin, thus genetic improvement will likely be slower than for other major species such as alfalfa. While breeding effort and resources are limited, a number of common breeding goals, such as improved winter survival, root and crown rot resistance, stand persistence, and high forage yield will continue to be important traits for facilitating wider adaptation of sainfoin. New breeding goals should also focus on the improvement of tolerance to frequent grazing and animal trampling to make this crop better suited for grazing. As the large seed size and cost of seed is a major limiting factor for adoption of sainfoin by farmers, breeding to improve seed yield without increasing seed size, or even by reducing seed size, without affecting other desirable traits would be beneficial.

In summary, this study quantified agro-morphological, nutritional traits and genetic diversity of 38 sainfoin accessions collected worldwide. The study established a relatively large database for many important traits of sainfoin for future breeding and improvement. The tested accessions were genetically diverse and superior accessions and individuals was identified. This study reveals correlations between important forage traits, such as forage DM yield with plant height, stem number, and day to flower, which will be significant for future selection. This research also revealed AFLP as a suitable marker to study genetic diversity of sainfoin.

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